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(57) Abstract

The invention relates to compositions and methods useful in the acceleration of binding of target analytes to capture ligands on surfaces. Detection proceeds through the use of an electron transfer moiety (ETM) that is associated with the target analyte, either directly or indirectly, to allow electronic detection of the ETM.

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/14191

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 98 20162 A (GOZIN MICHAEL ;YU CHANGJUN (US); KAYYEM JON F (US); CLINICAL MICRO) 14 May 1998 (1998-05-14) see whole doc. and esp. p.31 , 132 ff.	21-30 1-20
Y	US 4 787 963 A (MACCONNELL WILLIAM P) 29 November 1988 (1988-11-29) see esp. claims	1-20
A	US 5 015 569 A (PONTIUS BRIAN W) 14 May 1991 (1991-05-14) cited in the application	
A	WO 96 40712 A (CALIFORNIA INST OF TECHN) 19 December 1996 (1996-12-19) the whole document	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 December 1999	15/12/1999

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European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/14191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
A	OATABASE WPI Section Ch, Week 199803 Oerwent Publications Ltd., London, GB; Class B04, AN 1998-029208 XP002124777 "ONA detection for gene analysis in biological and medical application" & JP 09 288080 A (SHIN NIKKA KANKYO ENG KK), 4 November 1997 (1997-11-04) abstract	
P,X	WO 99 14596 A (BERGGREN CHRISTINE ;JOHANSSON GILLIS (SE); SANGTEC MEOICAL AB (SE)) 25 March 1999 (1999-03-25) see esp. claims	21-30

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 99/14191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820162 A	14-05-1998	AU 5196798 A	29-05-1998
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		US 5770369 A	23-06-1998
		US 5952172 A	14-09-1999
JP 9288080 A	04-11-1997	NONE	
WO 9914596 A	25-03-1999	AU 9099698 A	05-04-1999

The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to a nucleoside, with addition of additional nucleosides to form the capture probe followed by attachment to the electrode. Alternatively, the whole capture probe may be made and then the completed conductive oligomer added, followed by attachment to the electrode. Alternatively, a monolayer of conductive oligomer (some of which have functional groups for attachment of capture probes) is attached to the electrode first, followed by attachment of the capture probe. The latter two methods may be preferred when conductive oligomers are used which are not stable in the solvents and under the conditions used in traditional nucleic acid synthesis.

In a preferred embodiment, the compositions of the invention are made by first forming the conductive oligomer covalently attached to the nucleoside, followed by the addition of additional nucleosides to form a capture probe nucleic acid, with the last step comprising the addition of the conductive oligomer to the electrode.

The attachment of the conductive oligomer to the nucleoside may be done in several ways. In a preferred embodiment, all or part of the conductive oligomer is synthesized first (generally with a functional group on the end for attachment to the electrode), which is then attached to the nucleoside. Additional nucleosides are then added as required, with the last step generally being attachment to the electrode. Alternatively, oligomer units are added one at a time to the nucleoside, with addition of additional nucleosides and attachment to the electrode. A number of representative syntheses are shown in the Figures of WO 98/20162; PCT/US98/12430; PCT/US98/12082; PCT/US99/01705; PCT/US99/01703; and U.S.S.N.s 09/135,183; 60/105,875; and 09/295,691, all of which are incorporated by reference.

The conductive oligomer is then attached to a nucleoside that may contain one (or more) of the oligomer units, attached as depicted herein.

In a preferred embodiment, attachment is to a ribose of the ribose-phosphate backbone, including amide and amine linkages. In a preferred embodiment, there is at least a methylene group or other short aliphatic alkyl groups (as a Z group) between the nitrogen attached to the ribose and the aromatic ring of the conductive oligomer.

Alternatively, attachment is via a phosphate of the ribose-phosphate backbone, as generally outlined in PCT US97/20014.

In a preferred embodiment, attachment is via the base. In a preferred embodiment, protecting groups may be added to the base prior to addition of the conductive oligomers, as is generally known in the

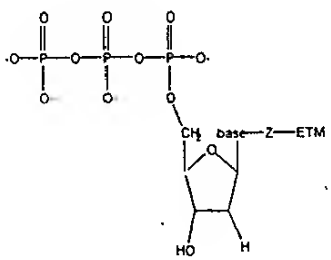
art In addition, the palladium cross-coupling reactions may be altered to prevent dimerization problems, i.e. two conductive oligomers dimerizing, rather than coupling to the base.

Alternatively, attachment to the base may be done by making the nucleoside with one unit of the oligomer, followed by the addition of others

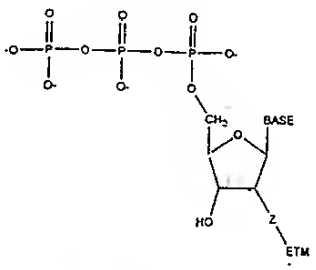
Once the modified nucleosides are prepared, protected and activated, prior to attachment to the electrode, they may be incorporated into a growing oligonucleotide by standard synthetic techniques (Gait, Oligonucleotide Synthesis A Practical Approach, IRL Press, Oxford, UK 1984; Eckstein) in several ways.

In one embodiment, one or more modified nucleosides are converted to the triphosphate form and incorporated into a growing oligonucleotide chain by using standard molecular biology techniques such as with the use of the enzyme DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Taq DNA polymerase, reverse transcriptase, and RNA polymerases. For the incorporation of a 3' modified nucleoside to a nucleic acid, terminal deoxynucleotidyltransferase may be used. (Ratliff, Terminal deoxynucleotidyltransferase. In The Enzymes, Vol 14A. P.D. Boyer ed. pp 105-118. Academic Press, San Diego, CA. 1981). Thus, the present invention provides deoxyribonucleoside triphosphates comprising a covalently attached ETM. Preferred embodiments utilize ETM attachment to the base or the backbone, such as the ribose (preferably in the 2' position), as is generally depicted below in Structures 42 and 43:

Structure 42



Structure 43



Thus, in some embodiments, it may be possible to generate the nucleic acids comprising ETMs in situ. For example, a target sequence can hybridize to a capture probe (for example on the surface) in such a way that the terminus of the target sequence is exposed, i.e. unhybridized. The addition of enzyme and triphosphate nucleotides labelled with ETMs allows the in situ creation of the label. Similarly, using labeled nucleotides recognized by polymerases can allow simultaneous PCR and detection; that is, the target sequences are generated in situ.

In a preferred embodiment, the modified nucleoside is converted to the phosphoramidite or H-phosphonate form, which are then used in solid-phase or solution syntheses of oligonucleotides. In this way the modified nucleoside, either for attachment at the nbose (i.e. amino- or thiol-modified nucleosides) or the base, is incorporated into the oligonucleotide at either an internal position or the 5' terminus. This is generally done in one of two ways. First, the 5' position of the ribose is protected with 4',4-dimethoxytrityl (DMT) followed by reaction with either 2-cyanoethoxy-bis-diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide, or by reaction with chlorodiisopropylamino 2'-cyanoethyoxyphosphine, to give the phosphoramidite as is known in the art, although other techniques may be used as will be appreciated by those in the art. See Gait, supra; Caruthers, Science 230:281 (1985), both of which are expressly incorporated herein by reference.

For attachment of a group to the 3' terminus, a preferred method utilizes the attachment of the modified nucleoside (or the nucleoside replacement) to controlled pore glass (CPG) or other oligomeric supports. In this embodiment, the modified nucleoside is protected at the 5' end with DMT, and then reacted with succinic anhydride with activation. The resulting succinyl compound is attached to CPG or other oligomeric supports as is known in the art. Further phosphoramidite nucleosides are added, either modified or not, to the 5' end after deprotection. Thus, the present invention provides conductive oligomers or insulators covalently attached to nucleosides attached to solid oligomeric supports such as CPG, and phosphoramidite derivatives of the nucleosides of the invention.

The invention further provides methods of making label probes with recruitment linkers comprising ETMs. These synthetic reactions will depend on the character of the recruitment linker and the

method of attachment of the ETM, as will be appreciated by those in the art. For nucleic acid recruitment linkers, the label probes are generally made as outlined herein with the incorporation of ETMs at one or more positions. When a transition metal complex is used as the ETM, synthesis may occur in several ways. In a preferred embodiment, the ligand(s) are added to a nucleoside, followed by the transition metal ion, and then the nucleoside with the transition metal complex attached is added to an oligonucleotide, i.e. by addition to the nucleic acid synthesizer. Alternatively, the ligand(s) may be attached, followed by incorportation into a growing oligonucleotide chain, followed by the addition of the metal ion.

In a preferred embodiment, ETMs are attached to a ribose of the ribose-phosphate backbone. This is generally done as is outlined herein for conductive oligomers, as described herein, and in PCT publication WO 95/15971, using amino-modified or oxo-modified nucleosides, at either the 2' or 3' position of the ribose. The amino group may then be used either as a ligand, for example as a transition metal ligand for attachment of the metal ion, or as a chemically functional group that can be used for attachment of other ligands or organic ETMs, for example via amide linkages, as will be appreciated by those in the art. For example, the examples describe the synthesis of nucleosides with a variety of ETMs attached via the ribose.

In a preferred embodiment, ETMs are attached to a phosphate of the ribose-phosphate backbone. As outlined herein, this may be done using phosphodiester analogs such as phosphoramidite bonds, see generally PCT publication WO 95/15971, or can be done in a similar manner to that described in PCT US97/20014, where the conductive oligomer is replaced by a transition metal ligand or complex or an organic ETM.

Attachment to alternate backbones, for example peptide nucleic acids or alternate phosphate linkages will be done as will be appreciated by those in the art.

In a preferred embodiment, ETMs are attached to a base of the nucleoside. This may be done in a variety of ways. In one embodiment, amino groups of the base, either naturally occurring or added as is described herein (see the figures, for example), are used either as ligands for transition metal complexes or as a chemically functional group that can be used to add other ligands, for example via an amide linkage, or organic ETMs. This is done as will be appreciated by those in the art. Alternatively, nucleosides containing halogen atoms attached to the heterocyclic ring are commercially available. Acetylene linked ligands may be added using the halogenated bases, as is generally known; see for example, Tzalis et al., Tetrahedron Lett. 36(34):6017-6020 (1995); Tzalis et al., Tetrahedron Lett. 36(2):3489-3490 (1995); and Tzalis et al., Chem. Communications (in press) 1996, all of which are hereby expressly incorporated by reference. See also the figures and the examples,

which describes the synthesis of metallocenes (in this case, ferrocene) attached via acetylene linkages to the bases.

In one embodiment, the nucleosides are made with transition metal ligands, incorporated into a nucleic acid, and then the transition metal ion and any remaining necessary ligands are added as is known in the art. In an alternative embodiment, the transition metal ion and additional ligands are added prior to incorporation into the nucleic acid.

Once the nucleic acids of the invention are made, with a covalently attached attachment linker (i.e. either an insulator or a conductive oligomer), the attachment linker is attached to the electrode. The method will vary depending on the type of electrode used. As is described herein, the attachment linkers are generally made with a terminal "A" linker to facilitate attachment to the electrode. For the purposes of this application, a sulfur-gold attachment is considered a covalent attachment.

In a preferred embodiment, conductive oligomers, insulators, and attachment linkers are covalently attached via sulfur linkages to the electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally not ideal for use in both synthesis of the compositions described herein and inclusion in oligonucleotide synthetic reactions. Accordingly, the present invention provides novel methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is depicted in the Figures. However, as will be appreciated by those in the art, when the conductive oligomers do not contain nucleic acids, traditional protecting groups such as acetyl groups and others may be used. See Greene et al., supra.

This may be done in several ways. In a preferred embodiment, the subunit of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.

This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. The protecting group is then removed and the sulfur-gold covalent attachment is made. Alternatively, all or part of the conductive oligomer is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. The conductive oligomer is then attached to a nucleoside, and additional

nucleosides attached. Alternatively, the conductive oligomer attached to a nucleic acid is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. Alternatively, the ethyl pyridine protecting group may be used as above, but removed after one or more steps and replaced with a standard protecting group like a disulfide. Thus, the ethyl pyridine or trimethylsilylethyl group may serve as the protecting group for some of the synthetic reactions, and then removed and replaced with a traditional protecting group.

By "subunit" of a conductive polymer herein is meant at least the moiety of the conductive oligomer to which the sulfur atom is attached, although additional atoms may be present, including either functional groups which allow the addition of additional components of the conductive oligomer, or additional components of the conductive oligomer. Thus, for example, when Structure 1 oligomers are used, a subunit comprises at least the first Y group.

A preferred method comprises 1) adding an ethyl pyridine or trimethylsilylethyl protecting group to a sulfur atom attached to a first subunit of a conductive oligomer, generally done by adding a vinyl pyridine or trimethylsilylethyl group to a sulfhydryl; 2) adding additional subunits to form the conductive oligomer; 3) adding at least a first nucleoside to the conductive oligomer; 4) adding additional nucleosides to the first nucleoside to form a nucleic acid; 5) attaching the conductive oligomer to the gold electrode. This may also be done in the absence of nucleosides, as is described in the Examples.

The above method may also be used to attach insulator molecules to a gold electrode.

In a preferred embodiment, a monolayer comprising conductive oligomers (and optionally insulators) is added to the electrode. Generally, the chemistry of addition is similar to or the same as the addition of conductive oligomers to the electrode, i.e. using a sulfur atom for attachment to a gold electrode, etc. Compositions comprising monolayers in addition to the conductive oligomers covalently attached to nucleic acids may be made in at least one of five ways: (1) addition of the monolayer, followed by subsequent addition of the attachment linker-nucleic acid complex; (2) addition of theattachment linker-nucleic acid complex followed by addition of the monolayer; (3) simultaneous addition of the monolayer and attachment linker-nucleic acid complex; (4) formation of a monolayer (using any of 1, 2 or 3) which includes attachment linkers which terminate in a functional moiety suitable for attachment of a completed nucleic acid; or (5) formation of a monolayer which includes attachment linkers which terminate in a functional moiety suitable for nucleic acid synthesis, i.e. the nucleic acid is synthesized on the surface of the monolayer as is known in the art. Such suitable functional moieties include, but are not limited to, nucleosides, amino groups, carboxyl groups, protected sulfur moieties, or hydroxyl

groups for phosphoramidite additions. The examples describe the formation of a monolayer on a gold electrode using the preferred method (1).

In a preferred embodiment, the nucleic acid is a peptide nucleic acid or analog. In this embodiment, the invention provides peptide nucleic acids with at least one covalently attached ETM or attachment linker. In a preferred embodiment, these moieties are covalently attached to an monomeric subunit of the PNA. By "monomeric subunit of PNA" herein is meant the -NH-CH_2CH_2-N($COCH_2$-Base)-CH_2-CO- monomer, or derivatives (herein included within the definition of "nucleoside") of PNA. For example, the number of carbon atoms in the PNA backbone may be altered; see generally Nielsen et al., Chem. Soc. Rev. 1997 page 73, which discloses a number of PNA derivatives, herein expressly incorporated by reference. Similarly, the amide bond linking the base to the backbone may be altered; phosphoramide and sulfuramide bonds may be used. Alternatively, the moieties are attached to an internal monomeric subunit. By "internal" herein is meant that the monomeric subunit is not either the N-terminal monomeric subunit or the C-terminal monomeric subunit. In this embodiment, the moieties can be attached either to a base or to the backbone of the monomeric subunit. Attachment to the base is done as outlined herein or known in the literature. In general, the moieties are added to a base which is then incorporated into a PNA as outlined herein. The base may be either protected, as required for incorporation into the PNA synthetic reaction, or derivatized, to allow incorporation, either prior to the addition of the chemical substituent or afterwards. Protection and derivatization of the bases is shown in PCT US97/20014. The bases can then be incorporated into monomeric subunits.

In a preferred embodiment, the moieties are covalently attached to the backbone of the PNA monomer. The attachment is generally to one of the unsubstituted carbon atoms of the monomeric subunit, preferably the α-carbon of the backbone, although attachment at either of the carbon 1 or 2 positions, or the α-carbon of the amide bond linking the base to the backbone may be done. In the case of PNA analogs, other carbons or atoms may be substituted as well. In a preferred embodiment, moieties are added at the α-carbon atoms, either to a terminal monomeric subunit or an internal one.

In this embodiment, a modified monomeric subunit is synthesized with an ETM or an attachment linker, or a functional group for its attachment, and then the base is added and the modified monomer can be incorporated into a growing PNA chain.

Once generated, the monomeric subunits with covalently attached moieties are incorporated into a PNA using the techniques outlined in Will et al., Tetrahedron 51(44):12069-12082 (1995), and Vanderlaan et al., Tett. Let. 38:2249-2252 (1997), both of which are hereby expressly incorporated in their entirety. These procedures allow the addition of chemical substituents to peptide nucleic acids without destroying the chemical substituents.

In an alternate preferred embodiment, an input electron source is used that has a higher redox potential than the ETM of the label probe. For example, luminol, an electron source, has a redox potential of roughly 720 mV. At voltages higher than the redox potential of the ETM, but lower than the redox potential of the electron source, i.e. 200 - 720 mV, the ferrocene is oxided, and transfers a single electron to the electrode via the conductive oligomer. However, the ETM is unable to accept any electrons from the luminol electron source, since the voltages are less than the redox potential of the luminol. However, at or above the redox potential of luminol, the luminol then transfers an electron to the ETM, allowing rapid and repeated electron transfer. In this way, the electron source (or co-reductant) serves to amplify the signal generated in the system, as the electron source molecules rapidly and repeatedly donate electrons to the ETM of the label probe.

Luminol has the added benefit of becoming a chemiluminiscent species upon oxidation (see Jirka et al., Analytica Chimica Acta 284:345 (1993)), thus allowing photo-detection of electron transfer from the ETM to the electrode. Thus, as long as the luminol is unable to contact the electrode directly, i.e. in the presence of the SAM such that there is no efficient electron transfer pathway to the electrode, luminol can only be oxidized by transferring an electron to the ETM on the label probe. When the ETM is not present, i.e. when the target sequence is not hybridized to the composition of the invention, luminol is not significantly oxidized, resulting in a low photon emission and thus a low (if any) signal from the luminol. In the presence of the target, a much larger signal is generated. Thus, the measure of luminol oxidation by photon emission is an indirect measurement of the ability of the ETM to donate electrons to the electrode. Furthermore, since photon detection is generally more sensitive than electronic detection, the sensitivity of the system may be increased. Initial results suggest that luminescence may depend on hydrogen peroxide concentration, pH, and luminol concentration, the latter of which appears to be non-linear.

Suitable electron source molecules are well known in the art, and include, but are not limited to, ferricyanide, and luminol.

Alternatively, output electron acceptors or sinks could be used, i.e. the above reactions could be run in reverse, with the ETM such as a metallocene receiving an electron from the electrode, converting it to the metallicenium, with the output electron acceptor then accepting the electron rapidly and repeatedly. In this embodiment, cobalticenium is the preferred ETM.

The presence of the ETMs at the surface of the monolayer can be detected in a variety of ways. A variety of detection methods may be used, including, but not limited to, optical detection (as a result of spectral changes upon changes in redox states), which includes fluorescence, phosphorescence, luminiscence, chemiluminescence, electrochemiluminescence, and refractive index; and electronic

detection, including, but not limited to, amperommetry, voltammetry, capacitance and impedence. These methods include time or frequency dependent methods based on AC or DC currents, pulsed methods, lock-in techniques, filtering (high pass, low pass, band pass), and time-resolved techniques including time-resolved fluoroscence.

In one embodiment, the efficient transfer of electrons from the ETM to the electrode results in stereotyped changes in the redox state of the ETM. With many ETMs including the complexes of ruthenium containing bipyridine, pyridine and imidazole rings, these changes in redox state are associated with changes in spectral properties. Significant differences in absorbance are observed between reduced and oxidized states for these molecules. See for example Fabbrizzi et al., Chem. Soc. Rev. 1995 pp197-202). These differences can be monitored using a spectrophotometer or simple photomultiplier tube device.

In this embodiment, possible electron donors and acceptors include all the derivatives listed above for photoactivation or initiation. Preferred electron donors and acceptors have characteristically large spectral changes upon oxidation and reduction resulting in highly sensitive monitoring of electron transfer. Such examples include $Ru(NH_3)_4py$ and $Ru(bpy)_2im$ as preferred examples. It should be understood that only the donor or acceptor that is being monitored by absorbance need have ideal spectral characteristics.

In a preferred embodiment, the electron transfer is detected fluorometrically. Numerous transition metal complexes, including those of ruthenium, have distinct fluorescence properties. Therefore, the change in redox state of the electron donors and electron acceptors attached to the nucleic acid can be monitored very sensitively using fluorescence, for example with Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$. The production of this compound can be easily measured using standard fluorescence assay techniques. For example, laser induced fluorescence can be recorded in a standard single cell fluorimeter, a flow through "on-line" fluorimeter (such as those attached to a chromatography system) or a multi-sample "plate-reader" similar to those marketed for 96-well immuno assays.

Alternatively, fluorescence can be measured using fiber optic sensors with nucleic acid probes in solution or attached to the fiber optic. Fluorescence is monitored using a photomultiplier tube or other light detection instrument attached to the fiber optic. The advantage of this system is the extremely small volumes of sample that can be assayed.

In addition, scanning fluorescence detectors such as the FluorImager sold by Molecular Dynamics are ideally suited to monitoring the fluorescence of modified nucleic acid molecules arrayed on solid

surfaces. The advantage of this system is the large number of electron transfer probes that can be scanned at once using chips covered with thousands of distinct nucleic acid probes.

Many transition metal complexes display fluorescence with large Stokes shifts. Suitable examples include bis- and trisphenanthroline complexes and bis- and trisbipyridyl complexes of transition metals such as ruthenium (see Juris, A., Balzani, V., et. al. Coord. Chem. Rev., V. 84, p. 85-277, 1988). Preferred examples display efficient fluorescence (reasonably high quantum yields) as well as low reorganization energies. These include Ru(4,7-biphenyl$_2$-phenanthroline)$_3^{2+}$, Ru(4,4′-diphenyl-2,2′-bipyridine)$_3^{2+}$ and platinum complexes (see Cummings et al., J. Am. Chem. Soc. 118:1949-1960 (1996), incorporated by reference). Alternatively, a *reduction* in fluorescence associated with hybridization can be measured using these systems.

In a further embodiment, electrochemiluminescence is used as the basis of the electron transfer detection. With some ETMs such as $Ru^{2+}(bpy)_3$, direct luminescence accompanies excited state decay. Changes in this property are associated with nucleic acid hybridization and can be monitored with a simple photomultiplier tube arrangement (see Blackburn, G. F. *Clin. Chem.* **37**: 1534-1539 (1991); and Juris et al., supra.

In a preferred embodiment, electronic detection is used, including amperommetry, voltammetry, capacitance, and impedence. Suitable techniques include, but are not limited to, electrogravimetry; coulometry (including controlled potential coulometry and constant current coulometry); voltametry (cyclic voltametry, pulse voltametry (normal pulse voltametry, square wave voltametry, differential pulse voltametry, Osteryoung square wave voltametry, and coulostatic pulse techniques); stripping analysis (aniodic stripping analysis, cathiodic stripping analysis, square wave stripping voltammetry); conductance measurements (electrolytic conductance, direct analysis); time-dependent electrochemical analyses (chronoamperometry, chronopotentiometry, cyclic chronopotentiometry and amperometry, AC polography, chronogalvametry, and chronocoulometry); AC impedance measurement; capacitance measurement; AC voltametry; and photoelectrochemistry.

In a preferred embodiment, monitoring electron transfer is via amperometric detection. This method of detection involves applying a potential (as compared to a separate reference electrode) between the nucleic acid-conjugated electrode and a reference (counter) electrode in the sample containing target genes of interest. Electron transfer of differing efficiencies is induced in samples in the presence or absence of target nucleic acid; that is, the presence or absence of the target nucleic acid, and thus the label probe, can result in different currents.

The device for measuring electron transfer amperometrically involves sensitive current detection and includes a means of controlling the voltage potential, usually a potentiostat. This voltage is optimized with reference to the potential of the electron donating complex on the label probe. Possible electron donating complexes include those previously mentioned with complexes of iron, osmium, platinum, cobalt, rhenium and ruthenium being preferred and complexes of iron being most preferred.

In a preferred embodiment, alternative electron detection modes are utilized. For example, potentiometric (or voltammetric) measurements involve non-faradaic (no net current flow) processes and are utilized traditionally in pH and other ion detectors. Similar sensors are used to monitor electron transfer between the ETM and the electrode. In addition, other properties of insulators (such as resistance) and of conductors (such as conductivity, impedance and capicitance) could be used to monitor electron transfer between ETM and the electrode. Finally, any system that generates a current (such as electron transfer) also generates a small magnetic field, which may be monitored in some embodiments.

It should be understood that one benefit of the fast rates of electron transfer observed in the compositions of the invention is that time resolution can greatly enhance the signal-to-noise results of monitors based on absorbance, fluorescence and electronic current. The fast rates of electron transfer of the present invention result both in high signals and stereotyped delays between electron transfer initiation and completion. By amplifying signals of particular delays, such as through the use of pulsed initiation of electron transfer and "lock-in" amplifiers of detection, and Fourier transforms.

In a preferred embodiment, electron transfer is initiated using alternating current (AC) methods. Without being bound by theory, it appears that ETMs, bound to an electrode, generally respond similarly to an AC voltage across a circuit containing resistors and capacitors. Basically, any methods which enable the determination of the nature of these complexes, which act as a resistor and capacitor, can be used as the basis of detection. Surprisingly, traditional electrochemical theory, such as exemplified in Laviron et al., J. Electroanal. Chem. 97:135 (1979) and Laviron et al., J. Electroanal. Chem. 105:35 (1979), both of which are incorporated by reference, do not accurately model the systems described herein, except for very small E_{AC} (less than 10 mV) and relatively large numbers of molecules. That is, the AC current (I) is not accurately described by Laviron's equation. This may be due in part to the fact that this theory assumes an unlimited source and sink of electrons, which is not true in the present systems.

The AC voltametry theory that models these systems well is outlined in O'Connor et al., J. Electroanal Chem. 466(2):197-202 (1999), hereby expressly incorporated by reference. The equation that predicts these systems is shown below as Equation 1:

Equation 1

$$I_{avg}=2nfFN_{total}\cdot\frac{\sinh[\frac{nF}{RT}\cdot E_{AC}]}{\cosh[\frac{nF}{RT}\cdot E_{AC}]+\cosh[\frac{nF}{RT}(E_{DC}-E_O)]}$$

In Equation 1, n is the number of electrons oxidized or reduced per redox molecule, f is the applied frequency, F is Faraday's constant, N_{total} is the total number of redox molecules, E_0 is the formal potential of the redox molecule, R is the gas constant, T is the temperature in degrees Kelvin, and E_{DC} is the electrode potential. The model fits the experimental data very well. In some cases the current is smaller than predicted, however this has been shown to be caused by ferrocene degradation which may be remedied in a number of ways.

In addition, the faradaic current can also be expressed as a function of time, as shown in Equation 2:

Equation 2

$$I_f(t)=\frac{q_e N_{total} nF}{2RT(\cosh[\frac{nF}{RT}(V(t)-E_0)]+1)}\cdot\frac{dV(t)}{dt}$$

I_F is the Faradaic current and q_e is the elementary charge

However, Equation 1 does not incorporate the effect of electron transfer rate nor of instrument factors. Electron transfer rate is important when the rate is close to or lower than the applied frequency. Thus, the true i_{AC} should be a function of all three, as depicted in Equation 3.

Equation 3

$$i_{AC} = f(\text{Nemst factors})f(k_{ET})f(\text{instrument factors})$$

These equations can be used to model and predict the expected AC currents in systems which use input signals comprising both AC and DC components. As outlined above, traditional theory surprisingly does not model these systems at all, except for very low voltages.

In general, non-specifically bound label probes/ETMs show differences in impedance (i.e. higher impedances) than when the label probes containing the ETMs are specifically bound in the correct orientation. In a preferred embodiment, the non-specifically bound material is washed away, resulting in an effective impedance of infinity. Thus, AC detection gives several advantages as is generally discussed below, including an increase in sensitivity, and the ability to "filter out" background noise. In

particular, changes in impedance (including, for example, bulk impedance) as between non-specific binding of ETM-containing probes and target-specific assay complex formation may be monitored

Accordingly, when using AC initiation and detection methods, the frequency response of the system changes as a result of the presence of the ETM. By "frequency response" herein is meant a modification of signals as a result of electron transfer between the electrode and the ETM. This modification is different depending on signal frequency. A frequency response includes AC currents at one or more frequencies, phase shifts, DC offset voltages, faradaic impedance, etc.

Once the assay complex including the target sequence and label probe is made, a first input electrical signal is then applied to the system, preferably via at least the sample electrode (containing the complexes of the invention) and the counter electrode, to initiate electron transfer between the electrode and the ETM. Three electrode systems may also be used, with the voltage applied to the reference and working electrodes. The first input signal comprises at least an AC component. The AC component may be of variable amplitude and frequency. Generally, for use in the present methods, the AC amplitude ranges from about 1 mV to about 1.1 V, with from about 10 mV to about 800 mV being preferred, and from about 10 mV to about 500 mV being especially preferred. The AC frequency ranges from about 0.01 Hz to about 100 MHz, with from about 10 Hz to about 10 MHz being preferred, and from about 100 Hz to about 20 MHz being especially preferred.

The use of combinations of AC and DC signals gives a variety of advantages, including surprising sensitivity and signal maximization.

In a preferred embodiment, the first input signal comprises a DC component and an AC component. That is, a DC offset voltage between the sample and counter electrodes is swept through the electrochemical potential of the ETM (for example, when ferrocene is used, the sweep is generally from 0 to 500 mV) (or alternatively, the working electrode is grounded and the reference electrode is swept from 0 to -500 mV). The sweep is used to identify the DC voltage at which the maximum response of the system is seen. This is generally at or about the electrochemical potential of the ETM. Once this voltage is determined, either a sweep or one or more uniform DC offset voltages may be used. DC offset voltages of from about -1 V to about +1.1 V are preferred, with from about -500 mV to about +800 mV being especially preferred, and from about -300 mV to about 500 mV being particularly preferred. In a preferred embodiment, the DC offset voltage is not zero. On top of the DC offset voltage, an AC signal component of variable amplitude and frequency is applied. If the ETM is present, and can respond to the AC perturbation, an AC current will be produced due to electron transfer between the electrode and the ETM.

For defined systems, it may be sufficient to apply a single input signal to differentiate between the presence and absence of the ETM (i.e. the presence of the target sequence) nucleic acid. Alternatively, a plurality of input signals are applied. As outlined herein, this may take a variety of forms, including using multiple frequencies, multiple DC offset voltages, or multiple AC amplitudes, or combinations of any or all of these.

Thus, in a preferred embodiment, multiple DC offset voltages are used, although as outlined above, DC voltage sweeps are preferred. This may be done at a single frequency, or at two or more frequencies.

In a preferred embodiment, the AC amplitude is varied. Without being bound by theory, it appears that increasing the amplitude increases the driving force. Thus, higher amplitudes, which result in higher overpotentials give faster rates of electron transfer. Thus, generally, the same system gives an improved response (i.e. higher output signals) at any single frequency through the use of higher overpotentials at that frequency. Thus, the amplitude may be increased at high frequencies to increase the rate of electron transfer through the system, resulting in greater sensitivity. In addition, this may be used, for example, to induce responses in slower systems such as those that do not possess optimal spacing configurations.

In a preferred embodiment, measurements of the system are taken at at least two separate amplitudes or overpotentials, with measurements at a plurality of amplitudes being preferred. As noted above, changes in response as a result of changes in amplitude may form the basis of identification, calibration and quantification of the system. In addition, one or more AC frequencies can be used as well.

In a preferred embodiment, the AC frequency is varied. At different frequencies, different molecules respond in different ways. As will be appreciated by those in the art, increasing the frequency generally increases the output current. However, when the frequency is greater than the rate at which electrons may travel between the electrode and the ETM, higher frequencies result in a loss or decrease of output signal. At some point, the frequency will be greater than the rate of electron transfer between the ETM and the electrode, and then the output signal will also drop.

In one embodiment, detection utilizes a single measurement of output signal at a single frequency. That is, the frequency response of the system in the absence of target sequence, and thus the absence of label probe containing ETMs, can be previously determined to be very low at a particular high frequency. Using this information, any response at a particular frequency, will show the presence of the assay complex. That is, any response at a particular frequency is characteristic of the assay

complex. Thus, it may only be necessary to use a single input high frequency, and any changes in frequency response is an indication that the ETM is present, and thus that the target sequence is present.

In addition, the use of AC techniques allows the significant reduction of background signals at any single frequency due to entities other than the ETMs, i.e. "locking out" or "filtering" unwanted signals. That is, the frequency response of a charge carrier or redox active molecule in solution will be limited by its diffusion coefficient and charge transfer coefficient. Accordingly, at high frequencies, a charge carrier may not diffuse rapidly enough to transfer its charge to the electrode, and/or the charge transfer kinetics may not be fast enough. This is particularly significant in embodiments that do not have good monolayers, i.e. have partial or insufficient monolayers, i.e. where the solvent is accessible to the electrode. As outlined above, in DC techniques, the presence of "holes" where the electrode is accessible to the solvent can result in solvent charge carriers "short circuiting" the system, i.e. the reach the electrode and generate background signal. However, using the present AC techniques, one or more frequencies can be chosen that prevent a frequency response of one or more charge carriers in solution, whether or not a monolayer is present. This is particularly significant since many biological fluids such as blood contain significant amounts of redox active molecules which can interfere with amperometric detection methods.

In a preferred embodiment, measurements of the system are taken at at least two separate frequencies, with measurements at a plurality of frequencies being preferred. A plurality of frequencies includes a scan. For example, measuring the output signal, e.g., the AC current, at a low input frequency such as 1 - 20 Hz, and comparing the response to the output signal at high frequency such as 10 - 100 kHz will show a frequency response difference between the presence and absence of the ETM. In a preferred embodiment, the frequency response is determined at at least two, preferably at least about five, and more preferably at least about ten frequencies.

After transmitting the input signal to initiate electron transfer, an output signal is received or detected. The presence and magnitude of the output signal will depend on a number of factors, including the overpotential/amplitude of the input signal; the frequency of the input AC signal; the composition of the intervening medium; the DC offset; the environment of the system; the nature of the ETM; the solvent, and the type and concentration of salt. At a given input signal, the presence and magnitude of the output signal will depend in general on the presence or absence of the ETM, the placement and distance of the ETM from the surface of the monolayer and the character of the input signal. In some embodiments, it may be possible to distinguish between non-specific binding of label probes and the formation of target specific assay complexes containing label probes, on the basis of impedance.

In a preferred embodiment, the output signal comprises an AC current. As outlined above, the magnitude of the output current will depend on a number of parameters. By varying these parameters, the system may be optimized in a number of ways.

In general, AC currents generated in the present invention range from about 1 femptoamp to about 1 milliamp, with currents from about 50 femptoamps to about 100 microamps being preferred, and from about 1 picoamp to about 1 microamp being especially preferred.

In a preferred embodiment, the output signal is phase shifted in the AC component relative to the input signal. Without being bound by theory, it appears that the systems of the present invention may be sufficiently uniform to allow phase-shifting based detection. That is, the complex biomolecules of the invention through which electron transfer occurs react to the AC input in a homogeneous manner, similar to standard electronic components, such that a phase shift can be determined. This may serve as the basis of detection between the presence and absence of the ETM, and/or differences between the presence of target-specific assay complexes comprising label probes and non-specific binding of the label probes to the system components.

The output signal is characteristic of the presence of the ETM; that is, the output signal is characteristic of the presence of the target-specific assay complex comprising label probes and ETMs. In a preferred embodiment, the basis of the detection is a difference in the faradaic impedance of the system as a result of the formation of the assay complex. Faradaic impedance is the impedance of the system between the electrode and the ETM. Faradaic impedance is quite different from the bulk or dielectric impedance, which is the impedance of the bulk solution between the electrodes. Many factors may change the faradaic impedance which may not effect the bulk impedance, and vice versa Thus, the assay complexes comprising the nucleic acids in this system have a certain faradaic impedance, that will depend on the distance between the ETM and the electrode, their electronic properties, and the composition of the intervening medium, among other things. Of importance in the methods of the invention is that the faradaic impedance between the ETM and the electrode is signficantly different depending on whether the label probes containing the ETMs are specifically or non-specifically bound to the electrode.

Accordingly, the present invention further provides electronic devices or apparatus for the detection of analytes using the compositions of the invention. The apparatus includes a test chamber for receiving a sample solution which has at least a first measuring or sample electrode, and a second measuring or counter electrode. Three electrode systems are also useful. The first and second measuring electrodes are in contact with a test sample receiving region, such that in the presence of a liquid test sample, the two electrophoresis electrodes may be in electrical contact.

In a preferred embodiment, the apparatus also includes detection electrodes comprising a single stranded nucleic acid capture probe covalently attached via an attachment linker, and a monolayer comprising conductive oligomers, such as are described herein.

The apparatus further comprises an AC voltage source electrically connected to the test chamber, that is, to the measuring electrodes. Preferably, the AC voltage source is capable of delivering DC offset voltage as well.

In a preferred embodiment, the apparatus further comprises a processor capable of comparing the input signal and the output signal. The processor is coupled to the electrodes and configured to receive an output signal, and thus detect the presence of the target nucleic acid.

Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings.

In a preferred embodiment, the probes are used in genetic diagnosis. For example, probes can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

In an additional embodiment, viral and bacterial detection is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, clymidia and other sexually transmitted diseases, may also be detected.

In a preferred embodiment, the nucleic acids of the invention find use as probes for toxic bacteria in the screening of water and food samples. For example, samples may be treated to lyse the bacteria to release its nucleic acid, and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*,

enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the probes are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the probes in an array are used for sequencing by hybridization.

Thus, the present invention provides for extremely specific and sensitive probes, which may, in some embodiments, detect target sequences without removal of unhybridized probe. This will be useful in the generation of automated gene probe assays.

Alternatively, the compositions of the invention are useful to detect successful gene amplification in PCR, thus allowing successful PCR reactions to be an indication of the presence or absence of a target sequence. PCR may be used in this manner in several ways. For example, in one embodiment, the PCR reaction is done as is known in the art, and then added to a composition of the invention comprising the target nucleic acid with a ETM, covalently attached to an electrode via a conductive oligomer with subsequent detection of the target sequence. Alternatively, PCR is done using nucleotides labelled with a ETM, either in the presence of, or with subsequent addition to, an electrode with a conductive oligomer and a target nucleic acid. Binding of the PCR product containing ETMs to the electrode composition will allow detection via electron transfer. Finally, the nucleic acid attached to the electrode via a conductive polymer may be one PCR primer, with addition of a second primer labelled with an ETM. Elongation results in double stranded nucleic acid with a ETM and electrode covalently attached. In this way, the present invention is used for PCR detection of target sequences.

In a preferred embodiment, the arrays are used for mRNA detection. A preferred embodiment utilizes either capture probes or capture extender probes that hybridize close to the 3' polyadenylation tail of the mRNAs. This allows the use of one species of target binding probe for detection, i.e. the probe contains a poly-T portion that will bind to the poly-A tail of the mRNA target. Generally, the probe will contain a second portion, preferably non-poly-T, that will bind to the detection probe (or other probe). This allows one target-binding probe to be made, and thus decreases the amount of different probe synthesis that is done.

In a preferred embodiment, the use of restriction enzymes and ligation methods allows the creation of "universal" arrays. In this embodiment, monolayers comprising capture probes that comprise restriction endonuclease ends, as is generally depicted in Figure 6. By utilizing complementary

portions of nucleic acid, while leaving "sticky ends", an array comprising any number of restriction endonuclease sites is made. Treating a target sample with one or more of these restriction endonucleases allows the targets to bind to the array. This can be done without knowing the sequence of the target. The target sequences can be ligated, as desired, using standard methods such as ligases, and the target sequence detected, using either standard labels or the methods of the invention.

The present invention provides methods which can result in sensitive detection of nucleic acids. In a preferred embodiment, less than about 10×10^6 molecules are detected, with less than about 10×10^5 being preferred, less than 10×10^4 being particularly preferred, less than about 10×10^3 being especially preferred, and less than about 10×10^2 being most preferred. As will be appreciated by those in the art, this assumes a 1:1 correlation between target sequences and reporter molecules; if more than one reporter molecule (i.e. electron transfer moeity) is used for each target sequence, the sensitivity will go up.

While the limits of detection are currently being evaluated, based on the published electron transfer rate through DNA, which is roughly 1×10^6 electrons/sec/duplex for an 8 base pair separation (see Meade et al., Angw. Chem. Eng. Ed., 34:352 (1995)) and high driving forces, AC frequencies of about 100 kHz should be possible. As the preliminary results show, electron transfer through these systems is quite efficient, resulting in nearly 100×10^3 electrons/sec, resulting in potential femptoamp sensitivity for very few molecules.

All references cited herein are incorporated by reference in their entireity.

EXAMPLES

Example 1

General Methods of Making Substrates and Monolayers

SAM formation on Substrates-General Procedure

The self-assembled monolayers were formed on a clean gold surface. The gold surface can be prepared by a variety of different methods: melted or polished gold wire, sputtered or evaporated gold on glass or mica or silicon wafers or some other substrate, electroplated or electroless gold on circuit board material or glass or silicon or some other substrate. Both the vacuum deposited gold samples (evaporated and sputtered) and the solution deposited gold samples (electroless and electroplated) often require the use of an adhesion layer between the substrate and the gold in order to insure good mechanical stability. Chromium, Titanium, Titanium/Tungsten or Tantalum is frequently employed with

sputtered and evaporated gold. Electroplated nickel is usually employed with electroplated and electroless gold, however other adhesion materials can be used.

The gold substrate is cleaned prior to monolayer formation. A variety of different procedures have been employed. Cleaning with a chemical solution is the most prevalent. Piranha solution (hydrogen peroxide/sulfuric acid) or aqua regia cleaning (Hydrochloric acid/ Nitric acid) is most prevalent, however electrochemical methods, flame treatment and plasma methods have also been employed.

Following cleaning, the gold substrate is incubated in a deposition solution. The deposition solution consists of a mixture of various thiols in a solvent. A mixture of alkane thiols in an organic solvent like ethanol is the most prevalent procedure, however numerous variations have been developed. Alternative procedures involve gas phase deposition of the alkane thiol, microcontact printing, deposition using neat thiol, deposition from aqueous solvent and two step procedures have been developed. The concentration of the alkane thiol in the deposition solution ranges from molar to submicromolar range with 0.5-2.0 millimolar being the most prevalent. The gold substrate is incubated/placed in contact with the deposition solution for less than a second to days depending on the procedure. The most common time is 1hr to overnight incubation. The incubation is usually performed at room temperature, however temperatures up to 50°C are common.

Mixed monolayers that contain DNA are usually prepared using a two step procedure. The thiolated DNA is deposited during the first deposition step and the mixed monolayer formation is completed during the second step in which a second thiol solution minus DNA is added. The second step frequently involves mild heating to promote monolayer reorganization.

General Procedure for SAM formation-Deposited from Organic Solution

A clean gold surface was placed into a clean vial. A DNA deposition solution in organic solvent was prepared in which the total thiol concentration was between 400 uM and 1.0 mM. The deposition solution contained thiol modified DNA and thiol diluent molecules. The ratio of DNA to diluent was usually between 10:1 and 1:10 with 1:1 being preferred. The preferred solvents are tetrahydrofuran (THF), acetonitrile, dimethylforamide (DMF) or mixtures thereof. Sufficient DNA deposition solution is added to the vial so as to completely cover the electrode surface. The gold substrate is allowed to incubate at ambient temperature or slightly above ambient temperature for 5-30 minutes. After the initial incubation, the deposition solution is removed and a solution of diluent molecule only (100 uM –1.0 mM) in organic solvent is added. The gold substrate is allowed to incubate at room temperature or above room temperature until a complete monolayer is formed (10 minutes-24 hours). The gold sample is removed from the solution, rinsed in clean solvent and used.

General Procedure for SAM formation-deposited from Aqueous Solution

A clean gold surface is placed into a clean vial. A DNA deposition solution in water is prepared in which the total thiol concentration is between 1 uM and 200 uM. The aqueous solution frequently has salt present (approximately 1M), however pure water can be used. The deposition solution contains thiol modified DNA and often a thiol diluent molecule. The ratio of DNA to diluent is usually between 10:1 and 1:10 with 1:1 being preferred. The DNA deposition solution is added to the vial in such a volume so as to completely cover the electrode surface. The gold substrate is allowed to incubate at ambient temperature or slightly above ambient temperature for 1-30 minutes with 5 minutes usually being sufficient. After the initial incubation, the deposition solution is removed and a solution of diluent molecule only (10 uM –1.0 mM) in either water or organic solvent is added. The gold substrate is allowed to incubate at room temperature or above room temperature until a complete monolayer is formed (10 minutes-24 hours). The gold sample is removed from the solution, rinsed in clean solvent and used.

Monolayers on Au Ball Electrodes

Creating Au Ball Electrodes: Use a razor blade to cut 10 cm lengths of gold wire (127 μm diameter, 99.99% pure; e.g. from Aldrich). Use a 16 gauge needle to pass the wire through a #4 natural rubber septum (of the size to fit over a ½ mL PCR eppendorf tube). (This serves to support the wire and seal the tubes during deposition. See below.) Use a clean-burning flame (methane or propane) to melt one centimeter of the wire and form a sphere attached to the wire terminus. Adjust the wire length such that when sealed in a PCR tube the gold ball would be positioned near the bottom, able to be submerged in 20 µL of liquid. On the day of use, dip the electrodes in aqua regia (4:3:1 H_2O:HCl:HNO_3) for 20 seconds and then rinse thoroughly with water.

Derivatization: For 5 minutes, heat 20 µL aliquots of deposition solutions (2:2:1 DNA/H6/M44 at 833 μM total in DMF) in PCR tubes on a PCR block at 50°C. Then put each electrode into a tube of deposition solution (submerging just the gold ball — as little of the wire "stem" as possible) and remove to room temperature. Incubate for fifteen minutes before transferring the electrodes into PCR tubes with 200 µL of 400 μM M44 in DMF (submerging much of the wire stem as well). Let sit in M44 at room temperature for 5 minutes, then put on the PCR block and run HCLONG. Take electrodes out of the M44 solution, dip in 6x SSC, and place in PCR tubes with 20 µL of hybridization solution. Dip electrodes in 6x SSC prior to ACV measurement.

HCLONG: 65°C 2', -0.3°C/s to 40°C, 40°C 2', +0.3°C/s to 55°C, 55°C 2', -0.3°C/s to 30°C, 30°C 2', +0.3°C/s to 35°C, 35°C 2', -0.3°C/s to 22°C

Manufacture of Circuit Boards

An 18" x 24" x 0.047" panel of FR-4 (General Electric) with a half-ounce copper foil on both sides was drilled according to specifications (Gerber files). The FR-4 panel is plated with electroless copper (500 microinches) to make the specified drill-holes conductive and then panel is plated with an additional 500 microinches of electroplated copper. Following copper plating, the panel is etched according to specifications via cupric chloride etching (acid etching). The etched panel is then plated with 400 microinches of electroplated nickel with brightner followed by 50 microinches of soft gold (99.99% purity). The gold panel is coated with liquid photoimagable solder mask (Probimer 52, Ciba-Geigy Co.) on both sides of the panel. The imaging is done according to specifications. 14 sensor electrodes that are 250 micron in diameter and 2 larger electrodes (500 microns in diameter) are created with insulated leads leading to gold plated contacts at the edge of the board. The solder masked panel is then scored according to specifications to create individual wafers that are 1" x 1". A silver/silver chloride paste is applied to one of the two larger electrodes (ERCON R-414). The panel is then plasma cleaned with an Argon/Oxygen Plasma mixture. Following cleaning, the panel is stored in a foil-lined bag until use.

Monolayer Deposition on Circuit Boards

The circuit boards are removed from the foil-lined bags and immersed in a 10% sulfuric acid solution for 30 seconds. Following the sulfuric acid treatment, the boards are immersed in two Milli-Q water baths for 1 minute each. The boards are then dried under a stream of nitrogen. The boards are placed on a X-Y table in a humidity chamber and a 30 nanoliter drop of DNA deposition solution is placed on each of the 14 electrodes. The DNA deposition solution consists of 33 uM thiolated DNA, 33 uM 2-unit phenylacetylene wire (H6), and 16 uM M44 in 6x SSC (900 mM sodium chloride, 90 mM sodium Citrate, pH 7) w/ 1% Triethylamine. The drop is incubated at room temperature for 5 minutes and then the drop is removed by rinsing in a Milli-Q water bath. The boards are immersed in a 45°C bath of M44 in acetontrile. After 30 minutes, the boards are removed and immersed in an acetonitrile bath for 30 seconds followed by a milli-Q water bath for 30 seconds. The boards are dried under a stream of nitrogen.

Example 2

Detection of Target Sequences

Monolayer Deposition on Circuit Boards

As above, the circuit boards were removed from the foil-lined bags and immersed in a 10% sulfuric acid solution for 30 seconds. Following the sulfuric acid treatment, the boards were immersed in two Milli-Q water baths for 1 minute each. The boards were then dried under a stream of nitrogen. The boards were placed on a X-Y table in a humidity chamber and a 30 nanoliter drop of DNA deposition solution was placed on each of the 14 electrodes. The DNA deposition solution consisted of 33 uM thiolated DNA, 33 uM 2-unit phenylacetylene wire (H6), and 16 uM undec-1-en-11yltri(ethylene

glycol)(HS-CH_2)$_{11}$-(OCH_2CH_2)$_3$-OH) in 6x SSC (900 mM sodium chloride, 90 mM sodium Citrate, pH 7) w/1% Triethylamine. 3 electrodes were spotted with a solution containing DNA 1 (5'-ACCATGGACACAGAT(CH_2)$_{16}$SH-3'). 4 electrodes were spotted with a solution containing DNA 2 (5'TCATTGATGGTCTCTTTTAACA((CH_2)$_{16}$SH-3'). 4 electrodes were spotted with DNA 3 (5'CACAGTGGGGGGACATCAAGCAGCCATGCAAA(CH_2)$_{16}$SH-3'). 3 electrodes were spotted with DNA 4 (5'-TGTGCAGTTGACGTGGAT(CH_2)$_{16}$SH-3') The deposition solution was allowed to incubate at room temperature for 5 minutes and then the drop was removed by rinsing in a Milli-Q water bath The boards were immersed in a 45°C bath of M44 in acetonitrile. After 30 minutes, the boards were removed and immersed in an acetonitrile bath for 30 seconds followed by a milli-Q water bath for 30 seconds. The boards were dried under a stream of nitrogen and stored in foiled-lined bags flushed with nitrogen until use.

Hybridization and Measurement

The modified boards were removed from the foil-lined bags and fitted with an injection molded sample chamber (cartridge). The chamber was adhered to the board using double-sided sticky tape and had a total volume of 250 microliters. A hybridization solution was prepared. The solution contains 10 nM DNA target (5'-TGTGCAGTTGACGTGGATTGTTAAAAGAGACCATCAATGAGGAAGCTGCA GAATGGGATAGAGTCATCCAGT-3' (D-998), 30 nM signaling probe (D-1055) and 10 nm 5'-TCTACAG(N6)C(N6)ATCTGTGTCCATGGT-3' (N6 is shown in Figure 1D of PCTUS99/01705; it comprises a ferrocene connected by a 4 carbon chain to the 2' oxygen of the ribose of a nucleoside) The signalling probe is as follows:

5'-(C23)$_4$-N87-N87-N87-N87-ATC CAC GTC AAC TGC ACA-3' (D- 1055)

| | | |
C23 C23 C23 C23
C23 C23 C23 C23
C23 C23 C23 C23
C23 C23 C23 C23

N87 is a branch point comprising a ring structure. C23 is shown in Figure 1F of PCTUS99/01705. In a solution containing 25% Qiagen lysis buffer AL, 455 mM $NaClO_4$, 195 mM NaCl, 1.0 mM mercaptohexanol and 10% fetal calf serum. 250 microliters of hybrid solution was injected into the cartridge and allowed to hybridize for 12 hours. After 12 hours, the hybridized chip was plugged into a homemade transconductance amplifier with switching circuitry. The transconductance amplifier was equipped with summing circuitry that combines a DC ramp from the computer DAQ card and an AC sine wave from the lock-in amplifier (SR830 Stanford Instruments). Each electrode was scanned sequentially and the data was saved and manipulated using a homemade program designed using Labview (National Instruments). The chip was scanned at between -100 mV and 500 mV (pseudo Ag/Ag/Cl reference electrode) DC with a 25 mV (50 mV peak to peak), 1000 Hz superimposed sine

wave. The output current was fed into the lock-in amplifier and the 1000 Hz signal was recorded (ACV technique). The data for each set of pads was compiled and averaged

	Ip	Relative Intensity Ip
DNA 1 (Positive 2 Fc)	34 nA	0.11
DNA 2 (Positive Sandwich Assay)	218 nA	0.7
DNA 3 (Negative)	0.3 nA	0.001
DNA 4 (Positive Sandwich Assay)	317 nA	1

The results are shown in Figure 14.

CLAIMS

We claim:

1. A method of detecting a target analyte in a sample comprising:

a) concentrating said target analyte in a detection chamber comprising a detection electrode comprising a covalently attached capture ligand;

b) binding said target analyte to said capture ligand to form an assay complex, wherein said assay complex further comprises at least one electron transfer moiety (ETM); and

c) detecting the presence of said ETM using said detection electrode.

2. A method according to claim 1 wherein said concentrating is done by placing said sample in an electric field between at least a first electrode and at least a second electrode sufficient to cause electrophoretic transport of said sample to said detection electrode.

3. A method according to claim 2 wherein said detection electrode is a porous electrode positioned between said first and said second electrodes.

4. A method according to claim 2 wherein said detection electrode is the same as said first electrode.

5. A method according to claim 2 wherein said detection electrode is separate from said first or second electrodes.

6. A method according to claim 4 wherein at least said first electrode comprises a permeation layer

7. A method according to claim 4 wherein an electroactive charge carrier is used for said electrophoretic transport.

8. A method according to claim 1 wherein said assay complex comprises a label probe comprising said ETM, and said label probe is positively charged.

9. A method according to claim 1 wherein said concentrating comprises including at least one volume exclusion agent in said detection chamber.

10. A method according to claim 1 wherein said concentrating comprises precipitating said target analyte.

11. A method according to claim 1 wherein said concentrating comprises including at least two reagents that form two separable solution phases, such that said target analyte concentrates in one of said phases.

12. A method according to claim 1 wherein said concentrating comprises binding said target analyte to a shuttle particle.

13. A method of detecting a target analyte in a sample comprising:

a) flowing said sample past a detection electrode comprising a covalently attached capture ligand under conditions that result in the formation of an assay complex comprising said target analyte and said capture ligand, wherein said assay complex further comprises at least one electron transfer moiety (ETM); and

c) detecting the presence of said ETM using said detection electrode.

14. A method according to claim 13 wherein the configuration of said detection electrode results in mixing of said sample.

15. A method according to claim 14 wherein said detection electrode comprises weirs.

16. A method according to claim 13 wherein said detection electrode is porous and is positioned such that said sample flows through said electrode.

17. A method of detecting a target nucleic acid sequence in a sample comprising:

a) indirectly or directly hybridizing said target sequence to a capture probe covalently attached to a detection electrode to form an assay complex, wherein said assay complex is formed in the presence of a hybridization accelerator, wherein said assay complex further comprises at least one electron transfer moiety (ETM); and

c) detecting the presence of said ETM using said detection electrode.

18. A method according to claim 7 wherein said hybridization accelerator is a nucleic acid binding protein.

19. A method according to claim 7 wherein said hybridization accelerator is a polyvalent ion.

20. A method of detecting a target analyte in a sample comprising:

a) adding said sample to a detection electrode comprising a covalently attached capture ligand under conditions that result in the formation of an assay complex comprising said target

analyte and said capture ligand, wherein said conditions include the presence of mixing particles, wherein said assay complex further comprises at least one electron transfer molety (ETM);

b) detecting the presence of said ETM using said detection electrode.

21. A substrate comprising a plurality of gold electrodes each comprising

a) a self-assembled monolayer;

b) a capture ligand; and

c) an interconnect such that each electrode is independently addressable.

22. A substrate according to claim 21 wherein said substrate is a printed circuit board material.

23. A substrate according to claim 22 wherein said printed circuit board material is fiberglass.

24. A substrate according to claim 21 wherein said substrate is plastic.

25. A method of making a substrate comprising a plurality of gold electrodes comprising:

a) coating an adhesion metal onto a fiberglass substrate;

b) coating gold onto said adhesion metal; and

c) forming a pattern comprising said plurality of electrodes and associated interconnects using lithography.

26. A method according to claim 25 further comprising adding a self-assembled monolayer (SAM) to each electrode.

27. A method according to claim 26 wherein said SAM comprises a species comprising a capture ligand.

28. A method according to claim 26 wherein said SAM is added using an aqueous deposition step.

29. A method of making a substrate comprising a plurality of gold electrodes comprising:

a) coating an adhesion metal onto said substrate;

b) coating gold onto said adhesion metal;

c) forming a pattern comprising said plurality of electrodes and associated interconnects using photolithography;

d) adding a self-assembled monolayer (SAM) comprising a capture ligand to each electrode.

30. A method according to claim 29 wherein said SAM is added using an aqueous deposition step

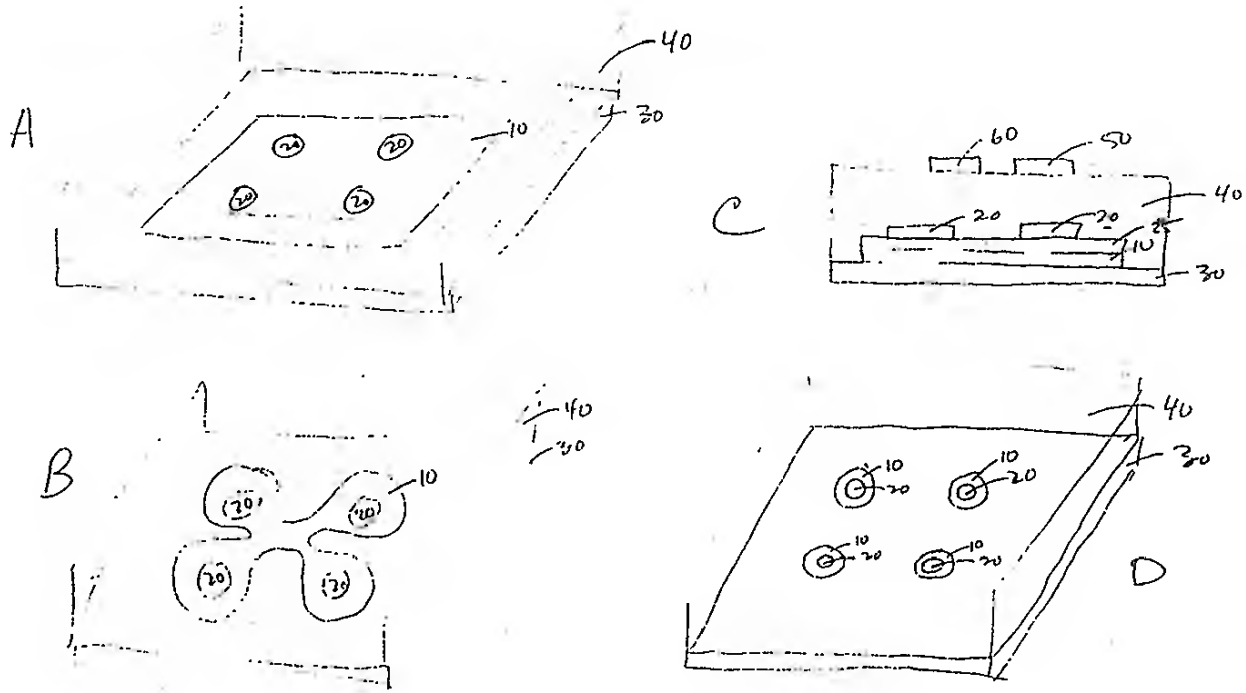

A
40
30
10
20
B
40
30
10
20
C
60
50
40
20
20
10
30
D
40
30
10
20
E
50
60
10
20
10
30

Fig 1

30
20
10
21
11
22
12
23
13

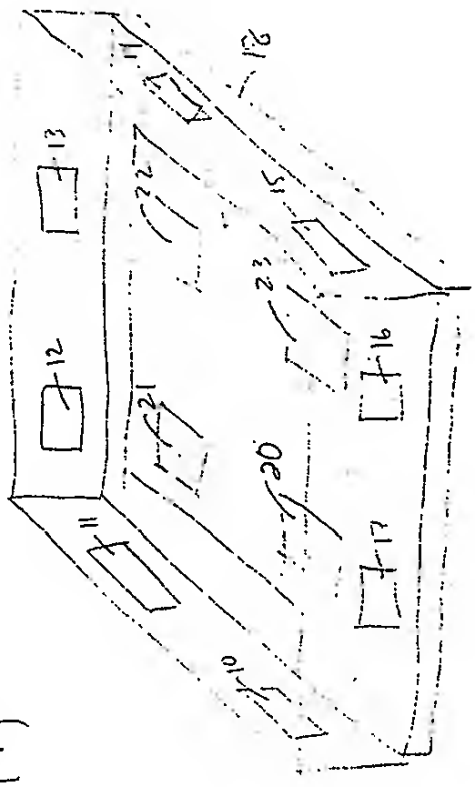
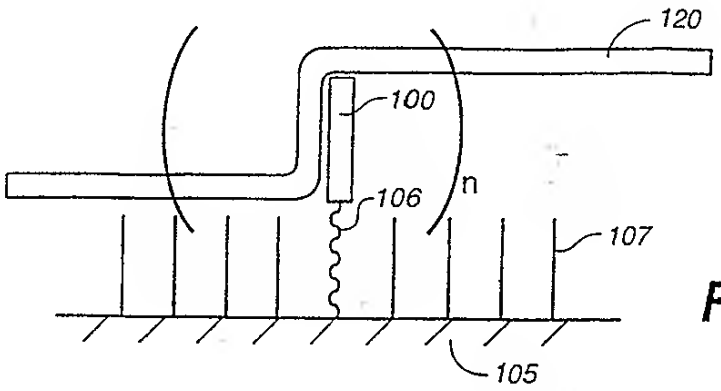
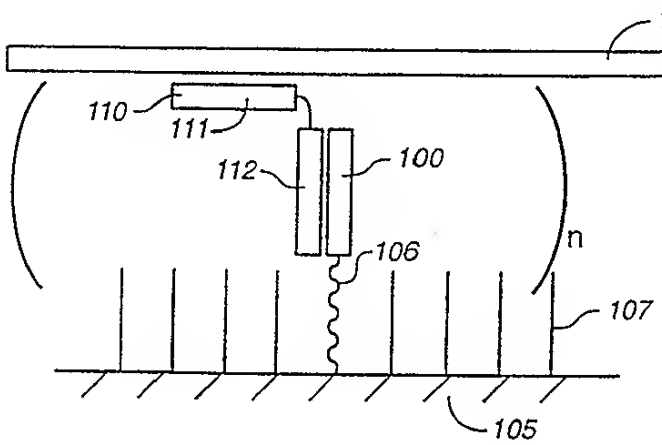


Fig 2



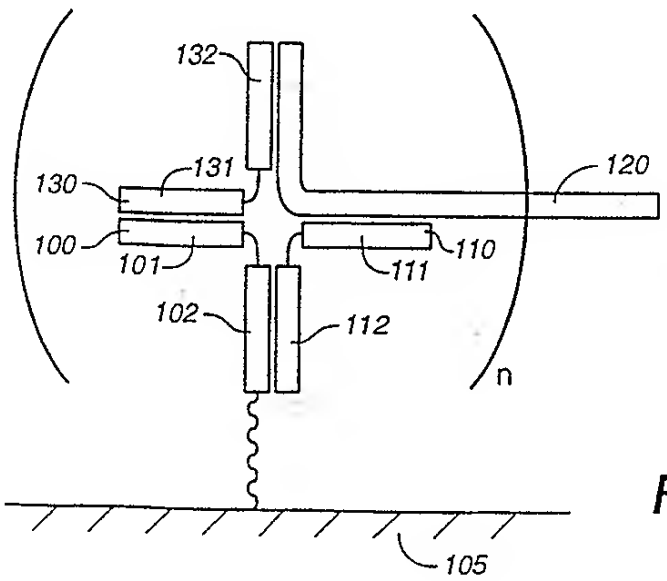
3A

FIG._1/5A



3

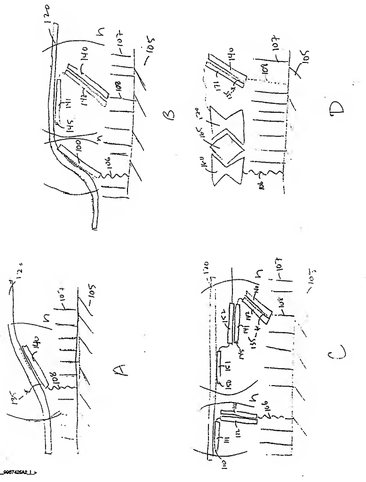
FIG._1/5B



3

FIG._1/5C

FIG 7



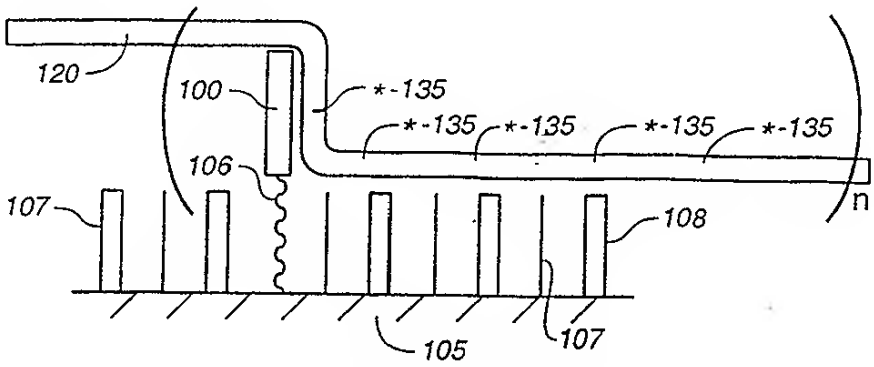


FIG._16A

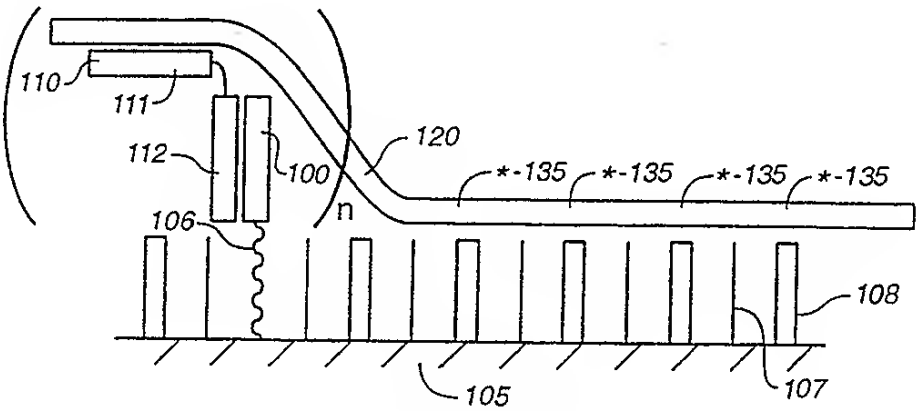


FIG._16B

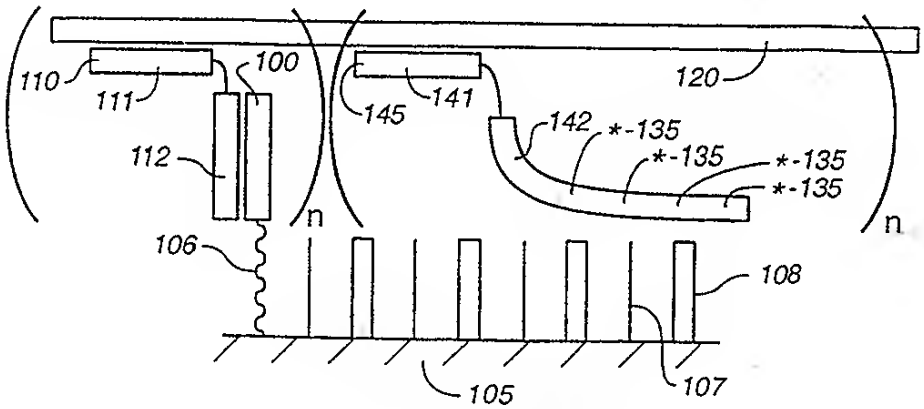


FIG._16C

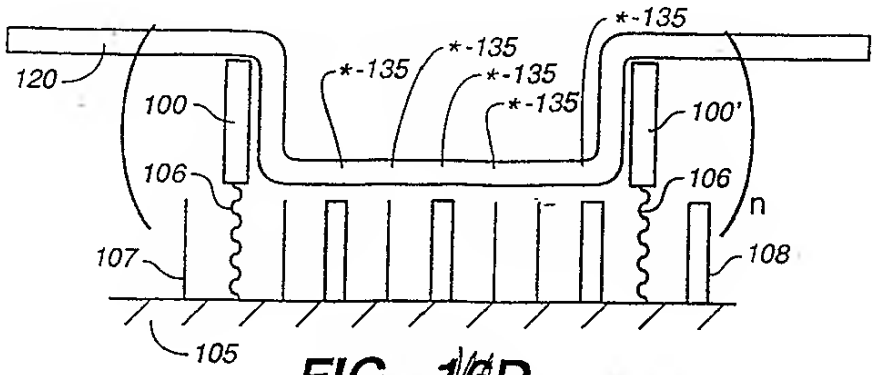


FIG._16D

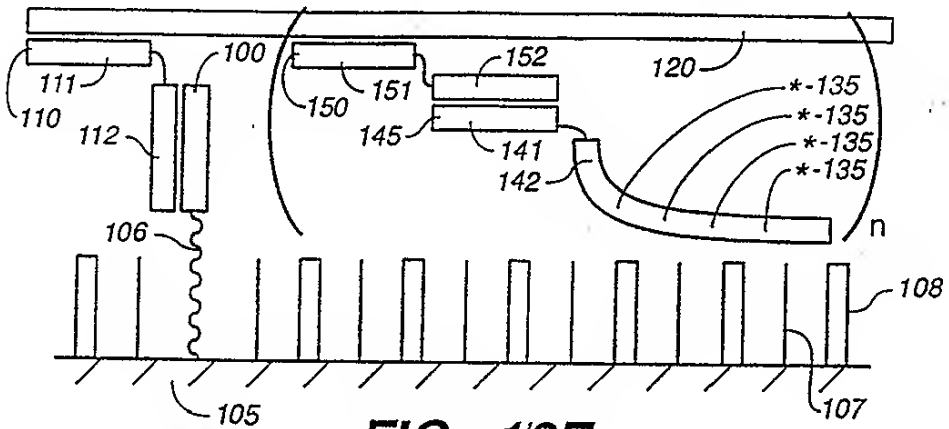


FIG._16E

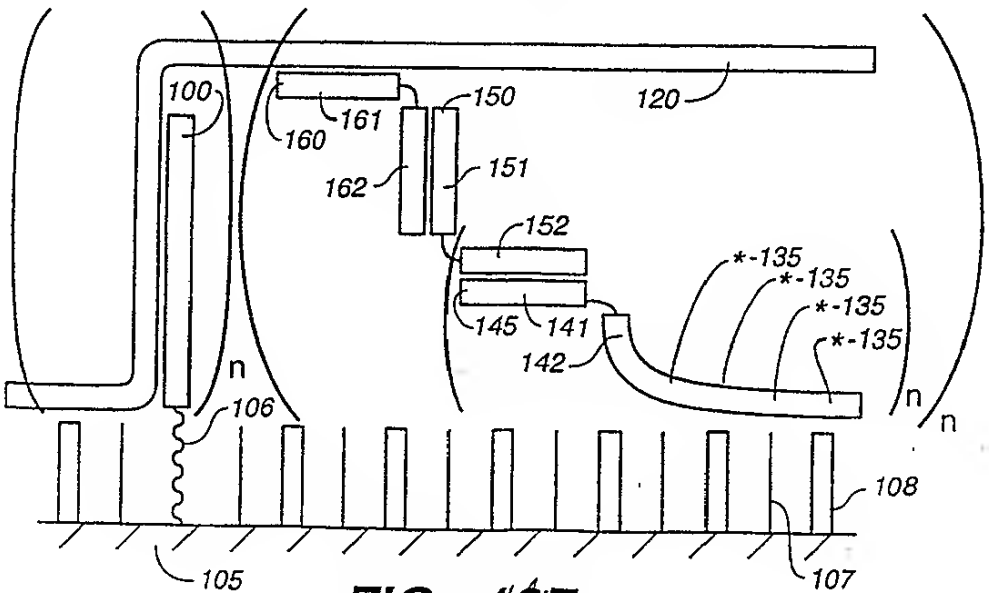


FIG._16F

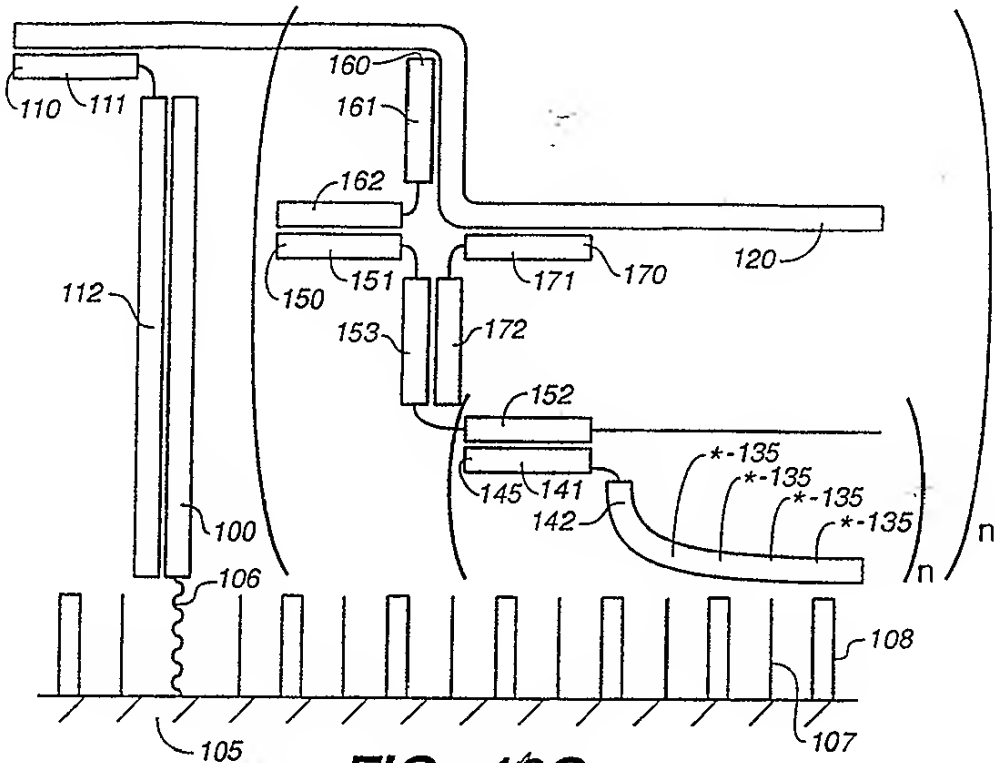


FIG._16G

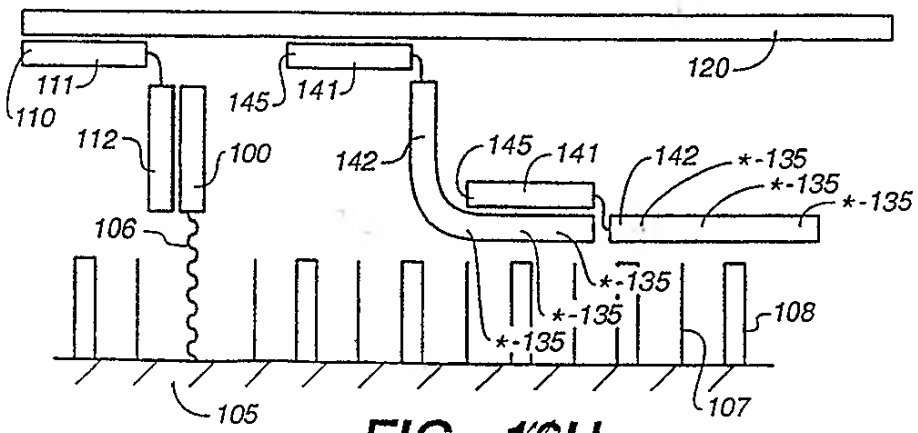
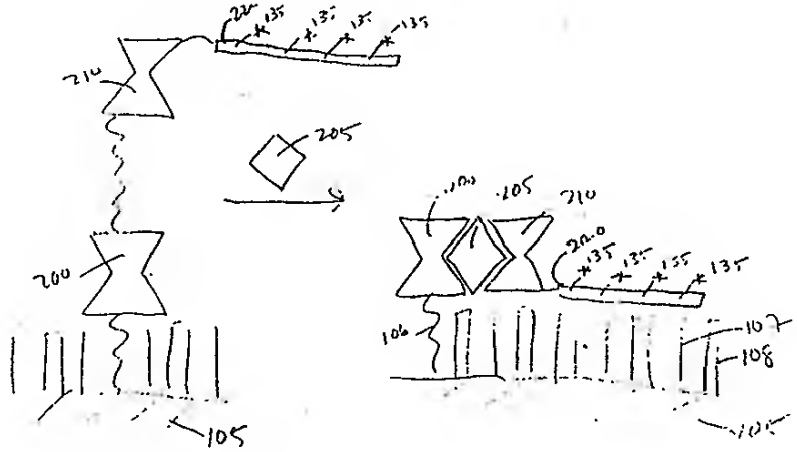
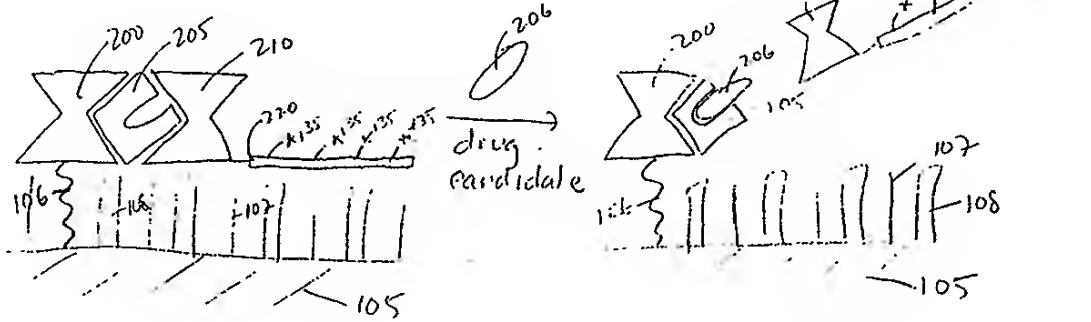


FIG._16H

Fig 9
A
B
C
D
E


220
135
135
135
135
210
205
200
105
100
105
210
200
135
135
135
135
106
107
108
105

F


200
205
210
220
135
135
135
135
106
107
105
206
drug
candidate
200
206
210
220
135
135
135
135
105
107
108
105

G

Fig 6
cont

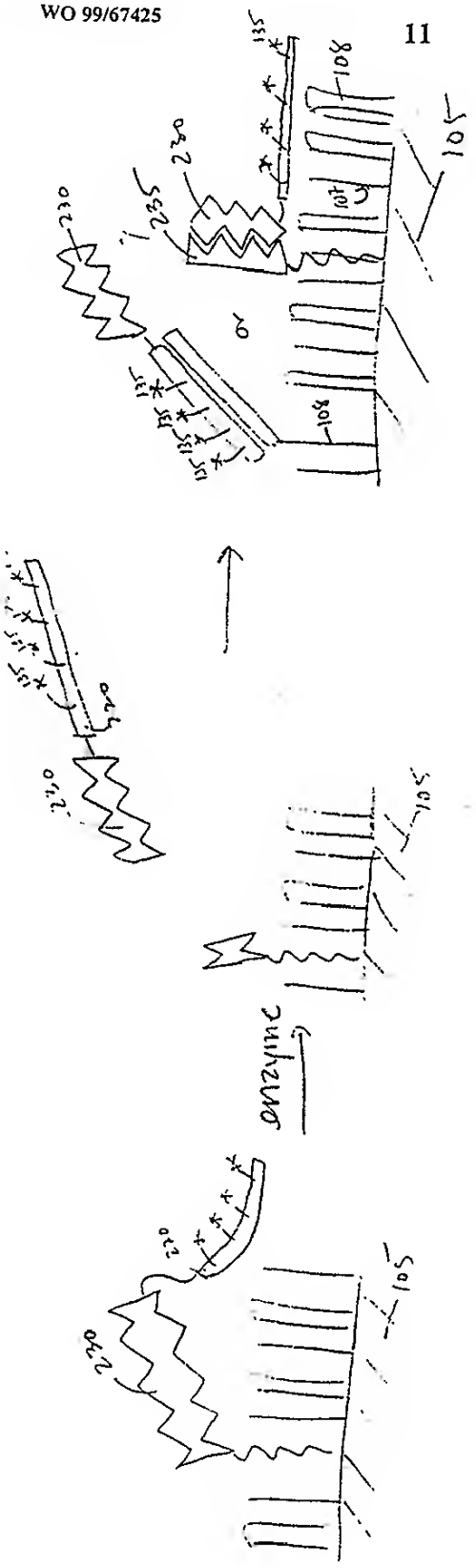
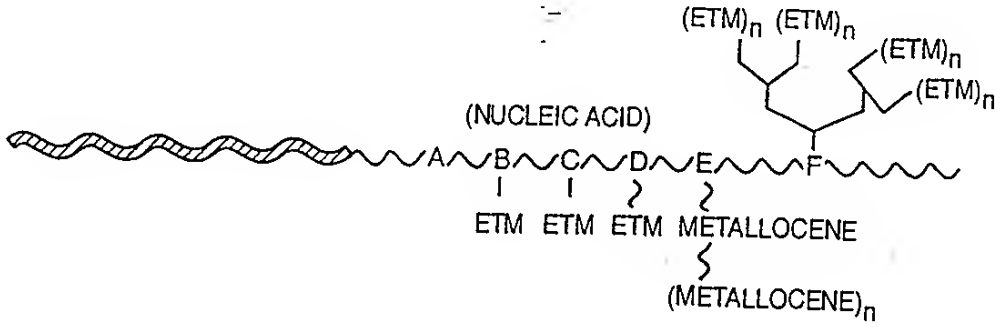


Fig. 6

= FIRST HYBRIDIZABLE PORTION OF LABEL PROBE

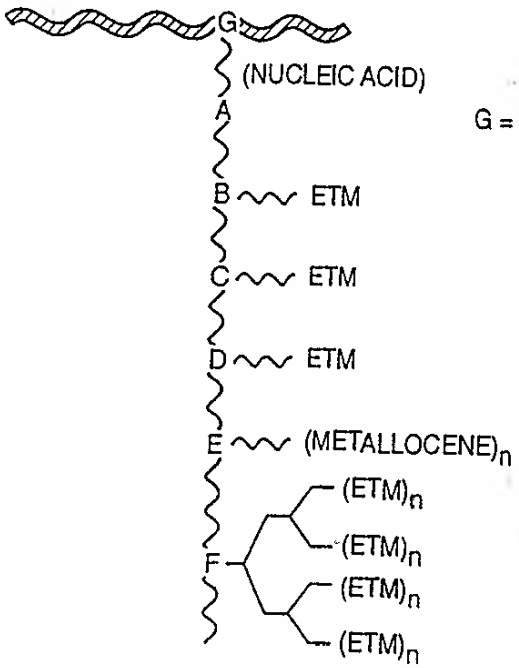
= RECRUITMENT LINKER



A = NUCLEOSIDE REPLACEMENT
B = ATTACHMENT TO A BASE
C = ATTACHEMENT TO A RIBOSE
D = ATTACHMENT TO A PHOSPHATE

E = METALLOCENE POLYMER, ATTACHED TO A RIBOSE, PHOSPHATE, OR BASE
F = DENDRIMER STRUCTURE, ATTACHED VIA A RIBOSE, PHOSPHATE OR BASE

FIG. 17A



G = ATTACHMENT VIA A "BRANCHING STRUCTURE", THROUGH RIBOSE, PHOSPHATE OR BASE

FIG. 17B

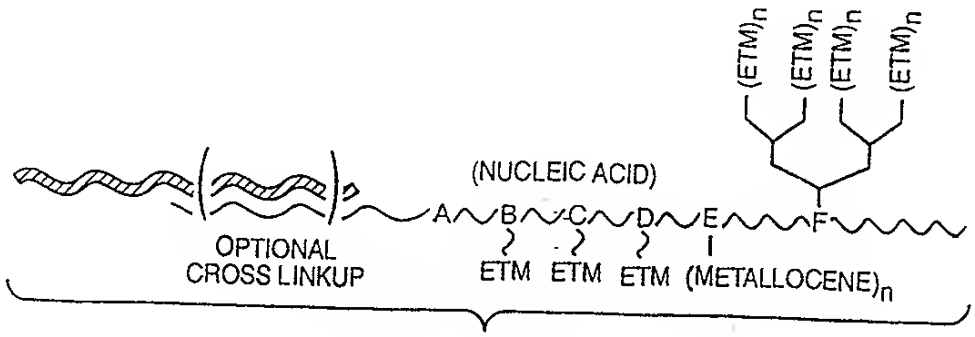


FIG. 17C

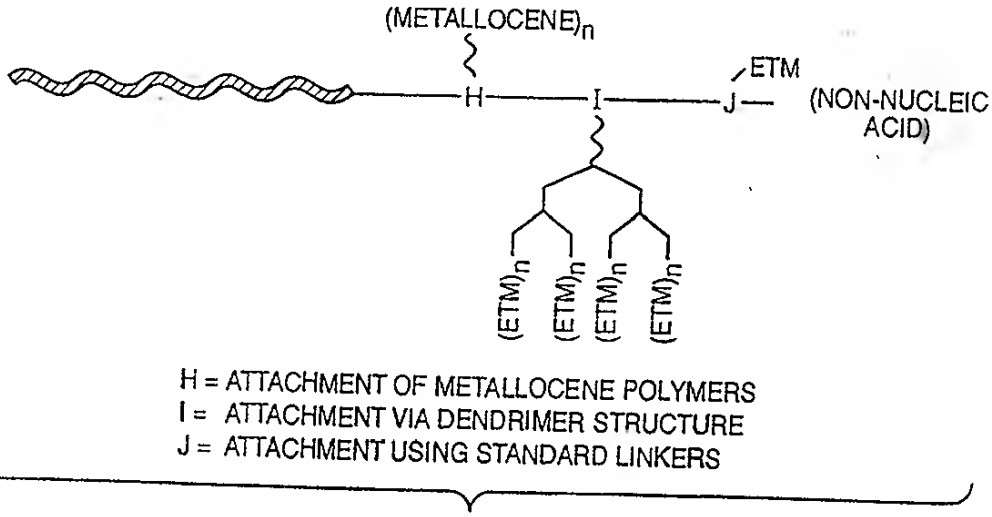


FIG. 17D

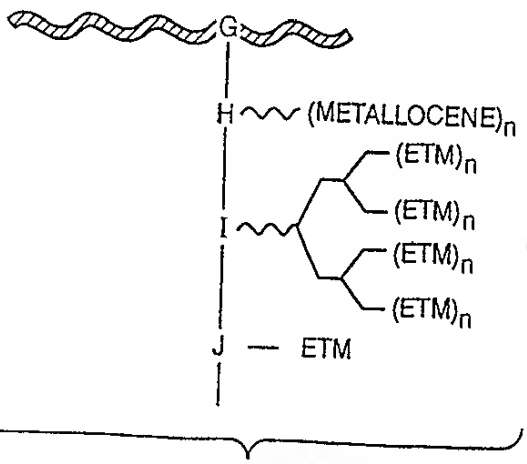


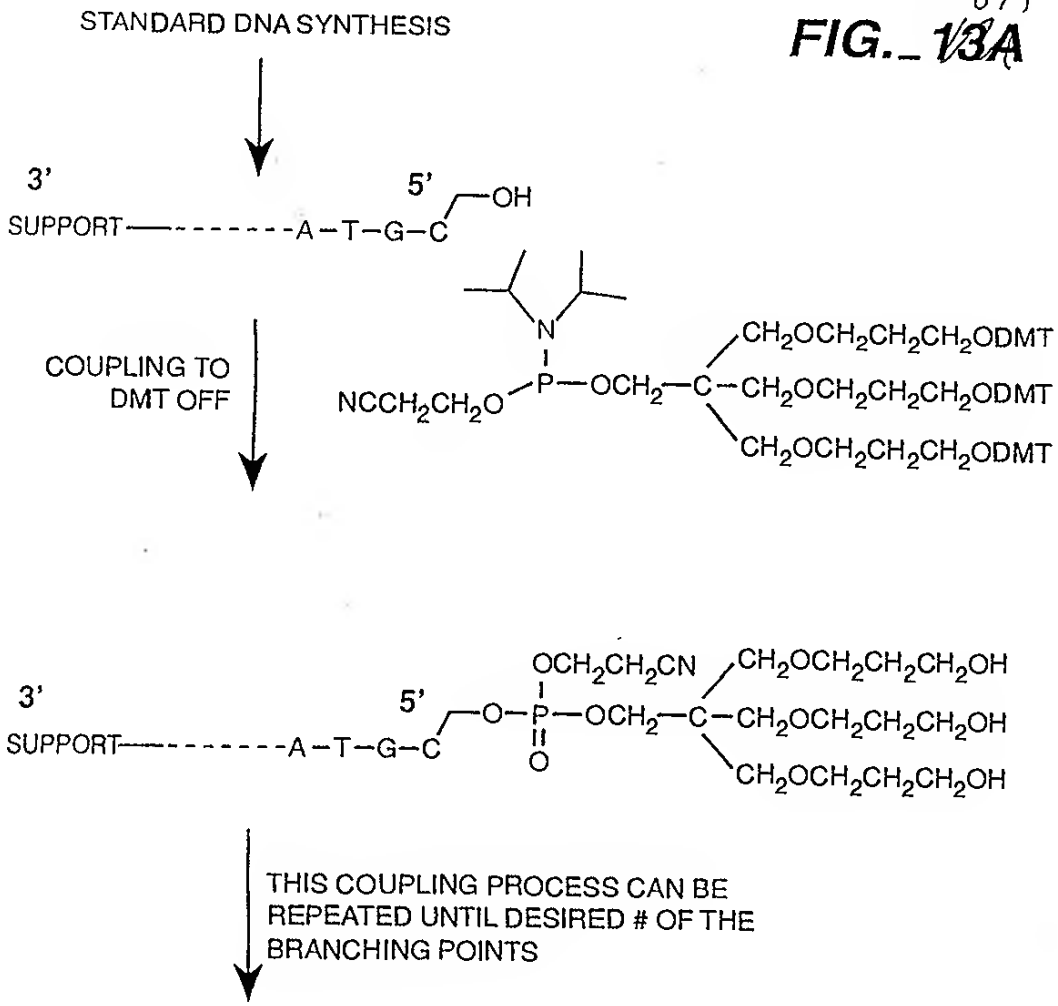
FIG. 17E

FIG._13

FIG._13A

FIG._13B

FIG._13A



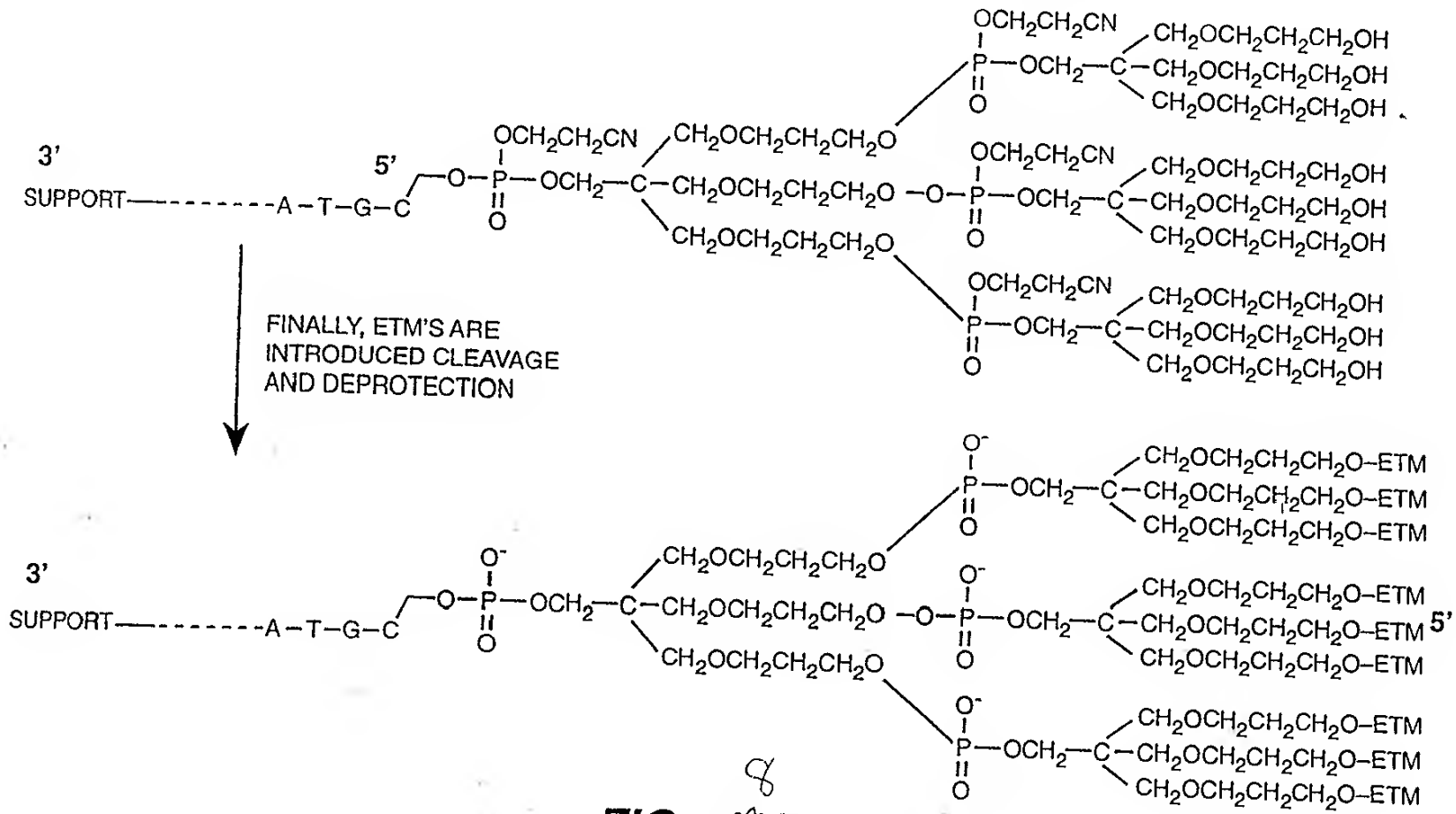

3'
5'
SUPPORT
A–T–G–C
OCH2CH2CN
CH2OCH2CH2CH2O
CH2OCH2CH2CH2OH
FINALLY, ETM'S ARE
INTRODUCED CLEAVAGE
AND DEPROTECTION
CH2OCH2CH2CH2O–ETM
5'

FIG._13B

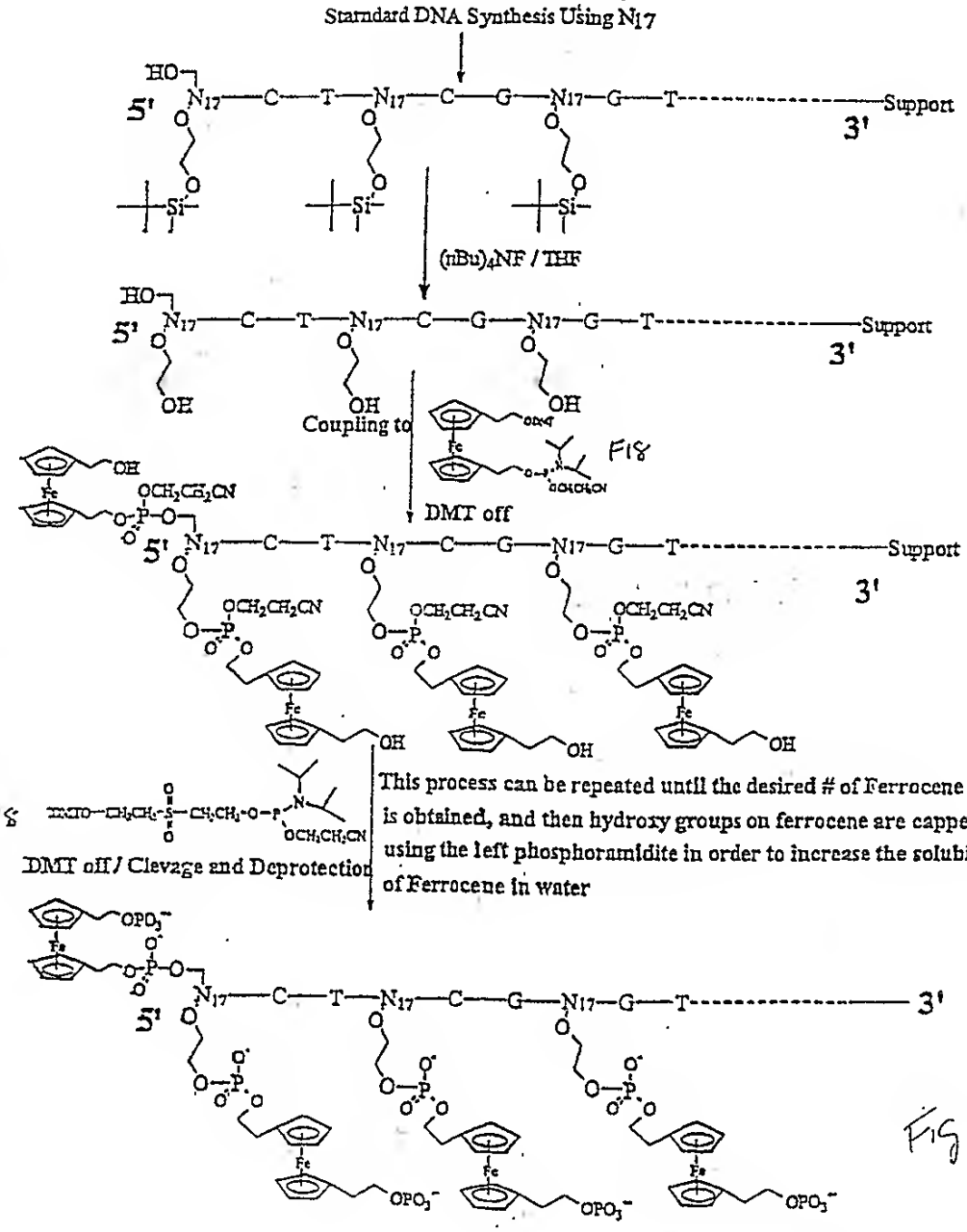

Starndard DNA Synthesis Using N17
HO
5'
N17—C—T—N17—C—G—N17—G—T
Support
3'
(nBu)4NF / THF
OH
Coupling to
Fig 8
DMT off
OCH2CH2CN
This process can be repeated until the desired # of Ferrocene is obtained, and then hydroxy groups on ferrocene are capped using the left phosphoramidite in order to increase the solubility of Ferrocene in water
DMT off / Clevage and Deprotection
OPO3-

Fig 9

Synthesis Scheme of Branched Adenosine

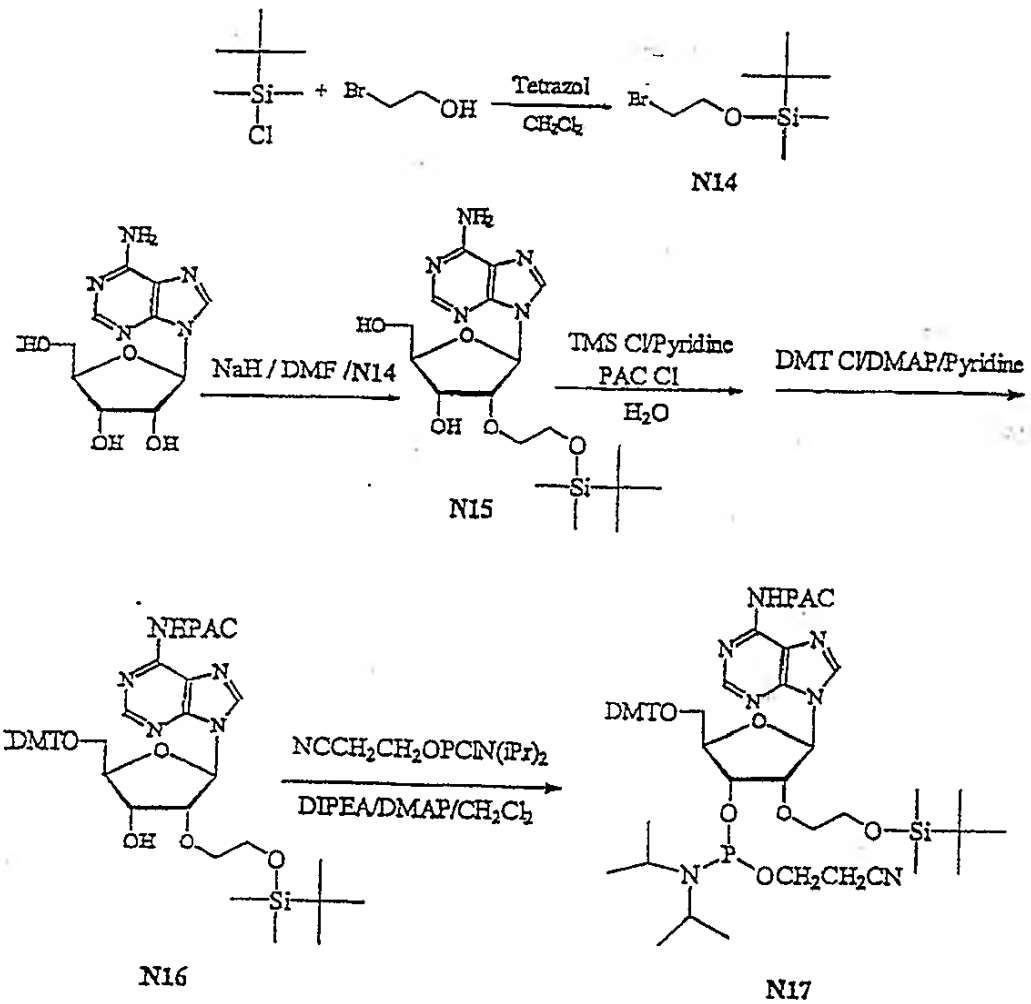


FIG. 10

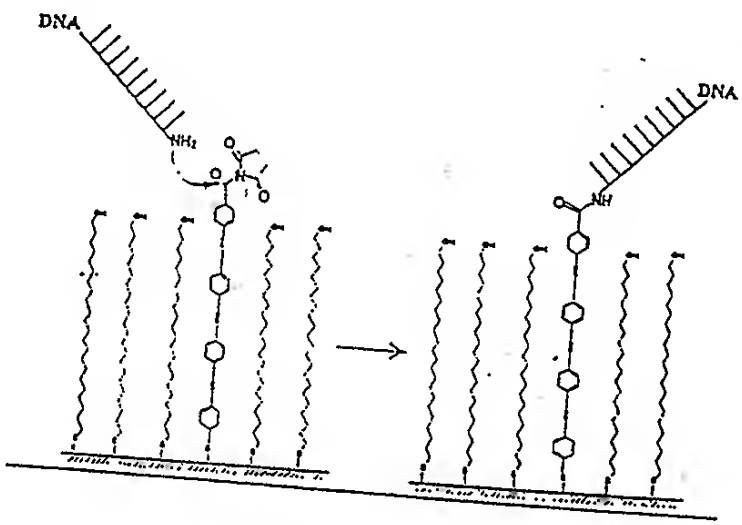


FIG. 11

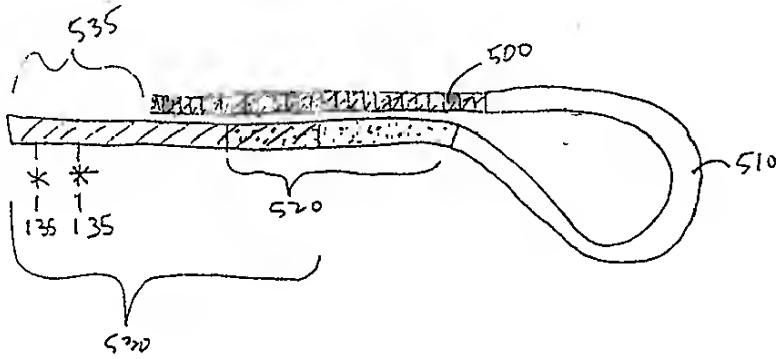


Fig 12

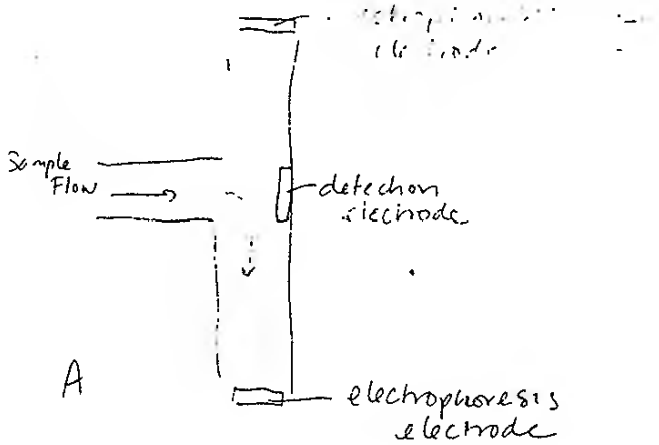
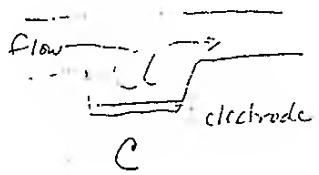
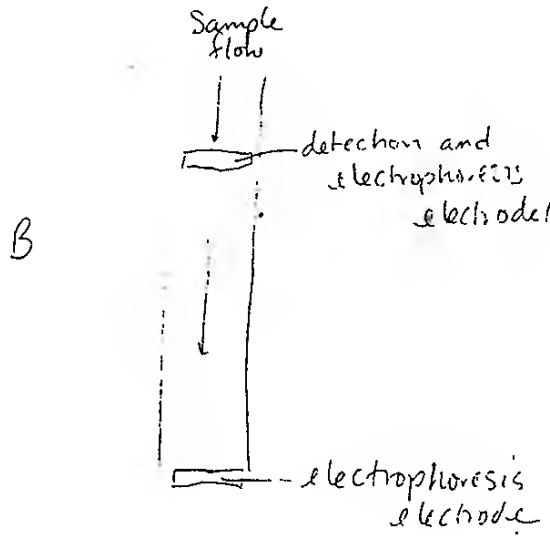
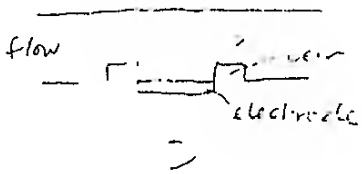

Sample Flow
detection electrode
electrophoresis electrode
A

Fig 13


flow
electrode
C


Sample flow
detection and electrophoresis electrode(s)
B
electrophoresis electrode


flow
electrode

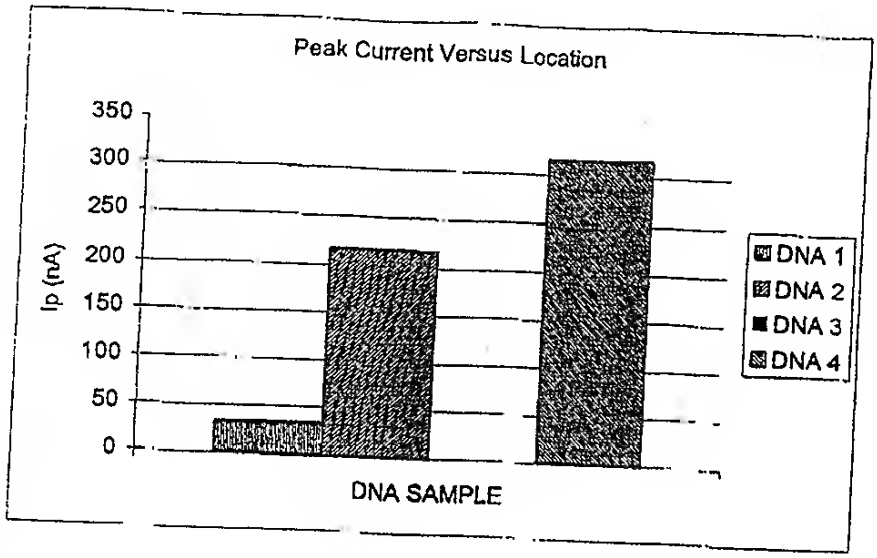

Peak Current Versus Location
350
300
250
200
150
100
50
0
Ip (nA)
DNA 1
DNA 2
DNA 3
DNA 4
DNA SAMPLE

Fig 14

INTERNATIONAL SEARCH REPORT

Information on patent family members

:rnational Application No
PCT/US 96/10702

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0478319	01-04-92	JP-A-	5199898	10-08-93
WO-A-9320230	14-10-93	GB-A,B	2280754	08-02-95
WO-A-8502627	20-06-85	AU-B-	569076	21-01-88
		AU-A-	2775384	08-11-84
		AU-B-	583258	27-04-89
		AU-A-	3832985	26-06-85
		CA-A-	1220818	21-04-87
		CA-A-	1223639	30-06-87
		EP-A-	0125139	14-11-84
		EP-A-	0149339	24-07-85
		JP-T-	61500706	17-04-86
		US-A-	4840893	20-06-89
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		EP-A-	0691978	17-01-96
		JP-T-	8508311	03-09-96
WO-A-9500530	05-01-95	AU-A-	7212494	17-01-95
		EP-A-	0705271	10-04-96
US-A-5272056	21-12-93	US-A-	5504075	02-04-96
WO-A-9115768	17-10-91	AU-A-	7575291	30-10-91
		CA-A-	2080019	07-10-91
		EP-A-	0523171	20-01-93

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 10702

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-137,148-151: A method for the detection of nucleic acid and apparatus therefor
- claims 138-142 : A microelectronic device
- claims 143-147 : A method for detecting a nucleic acid and oligonucleotide probe therefor

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-137,148-151

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees